VOLUME 45 NOVEMBER, 1955 NUMBER 11

UNIVERSITY OF MICHIGAN
NOV 17 1955
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Phytopathology

An International Journal

Official Organ of

THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

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EXPERIMENTAL TRANSMISSION OF THE OAK WILT FUNGUS BY CAGED SQUIRRELS [1]

E. B. Himelick and E. A. Curl

SUMMARY

Field observations made during the fall of 1953 and winter and spring of 1953-54 in Illinois gave evidence of an increase in the amount of rodent feeding, probably squirrel feeding, on mycelial mats of *Endoconidiophora fagacearum* Bretz.

Gray squirrels and fox squirrels were caged with potted healthy oak trees and given food contaminated with the oak wilt fungus. The squirrels were left to wound the trees of their own volition. They inflicted damage, by gnawing, on 89 of 209 trees used. None of the wounded 89 trees wilted, and *E. fagacearum* was not isolated from them.

Later, an experiment was conducted in the greenhouse in which captured gray squirrels and fox squirrels were used. The squirrels were fed conidium inoculum on apple, ascospore-conidium inoculum on apple, and conidium-bearing mycelial mats on oak bolts. They were then allowed or forced to make gnawing wounds on healthy potted oaks. Thirty-three per cent of the trees wounded by squirrels that fed on conidium inoculum, 37 per cent of those wounded by squirrels that fed on ascospore-conidium inoculum, and 33 per cent of those wounded by squirrels that fed on conidium-bearing mycelial mats developed oak wilt symptoms and died. The caged squirrels transmitted the oak wilt fungus to healthy potted oaks for intervals up to 12 hours when fed artificial inoculum and for a period of 1 hour when fed naturally occurring mycelial mats.

The combined results of greenhouse experiments and field observations strongly suggest that squirrels may occasionally transmit the oak wilt fungus.

Of several spray materials tested for their squirrel repelling qualities, only creosote prevented squirrel feeding on the subcortical fungus mats.

Buller (1) cited several field observations made by himself and others of squirrels eating and storing fruiting bodies of many of the higher fungi. Feeding by rodents, probably gray squirrels, on mycelial mats of *Endoconidiophora fagacearum* in Pennsylvania was reported by Morris and Fergus (6) in 1952. Himelick et al (4) observed extensive damage to the bark of mat-producing diseased oaks and of healthy oaks in Illinois forests during the winter of 1952-53 and obtained information which indicated that fox and gray squirrels are possible vectors of the oak wilt fungus. Feeding damage of this kind has since been seen in other states where oak wilt is prevalent.

For 16 months following the discovery of the subcortical fruiting mats of the oak wilt fungus in Illinois (3) in September of 1951, no evidence of feeding on the fungus by rodents was noticed. In December, 1952, and January, 1953, Himelick et al (4) found holes apparently gnawed by squirrels in the bark on 16 of 19 mat-bearing red and black oaks in 4 widely separated oak wilt areas in the state. Small areas of bark directly outside the fungus mats were completely torn away by the animals, and feeding usually was confined to the thick central pads or pressure cushions (Fig. 1, A). Only a few scattered mats on each tree were involved, while many other mats were left undisturbed. In the first survey of the Sinnissippi Forest area, a 1600-acre stand of native timber near Oregon, Illinois, evidence of squirrel damage was observed on 10 of 12 mat-bearing trees. This forest was surveyed again during the fall of 1953, the winter and spring of 1953-54, and the winter and spring of 1954-55. During these periods, feeding damage to mats was found on 45 of 56 mat-bearing trees. Most of the damage occurred during the late winter and spring months; however, some feeding occurred during the fall of 1953 and of 1954. As many as 65 holes in the bark were counted on a single tree, and the damage occurred on trees ranging in size from 1 to 30 in. d.b.h. (diameter at breast height). Unlike the first damage seen in the state, this later damage often involved every mat on a tree (Fig. 1, B). Also, the holes were not always confined to areas covering the central pads but often exposed entire mats (Fig. 1, C). Numerous paired tooth-marks on the outer wood provided evidence that the squirrels had eaten not only the thick pads but also the surrounding loose mycelium. Holes often were found in the bark in places where no mats existed. This observation suggested that squirrels were attracted by the odor that is present in wood and bark of wilt-killed trees prior to mat formation.

INITIAL TRANSMISSION TESTS.—Between March 1, and July 1, 1953, an experiment was carried on to determine if caged squirrels could transmit the oak wilt fungus. Six wire-screen cages measuring 10 × 3 × 4 ft. were arranged outdoors in shade. Each cage contained a small nesting hutch. One squirrel and 20 potted 3-year-old red oaks, *Quercus borealis* Michx. f., were placed in each cage. Two fox squirrels, *Scuirus niger*, and 4 gray squirrels, *Scuirus carolinensis*, were used in the experiment. The inoculum consisted of 2- to 3-week old cultures of *E. fagacearum* grown in the laboratory on moist wheat bran. The cultures contained endoconidia and much thick pad material similar to that which occurs in nature. At 2-day intervals, some of these cultures were mixed with water in a Waring Blendor to make a thick suspension of spores,

[1] Accepted for publication June 27, 1955.

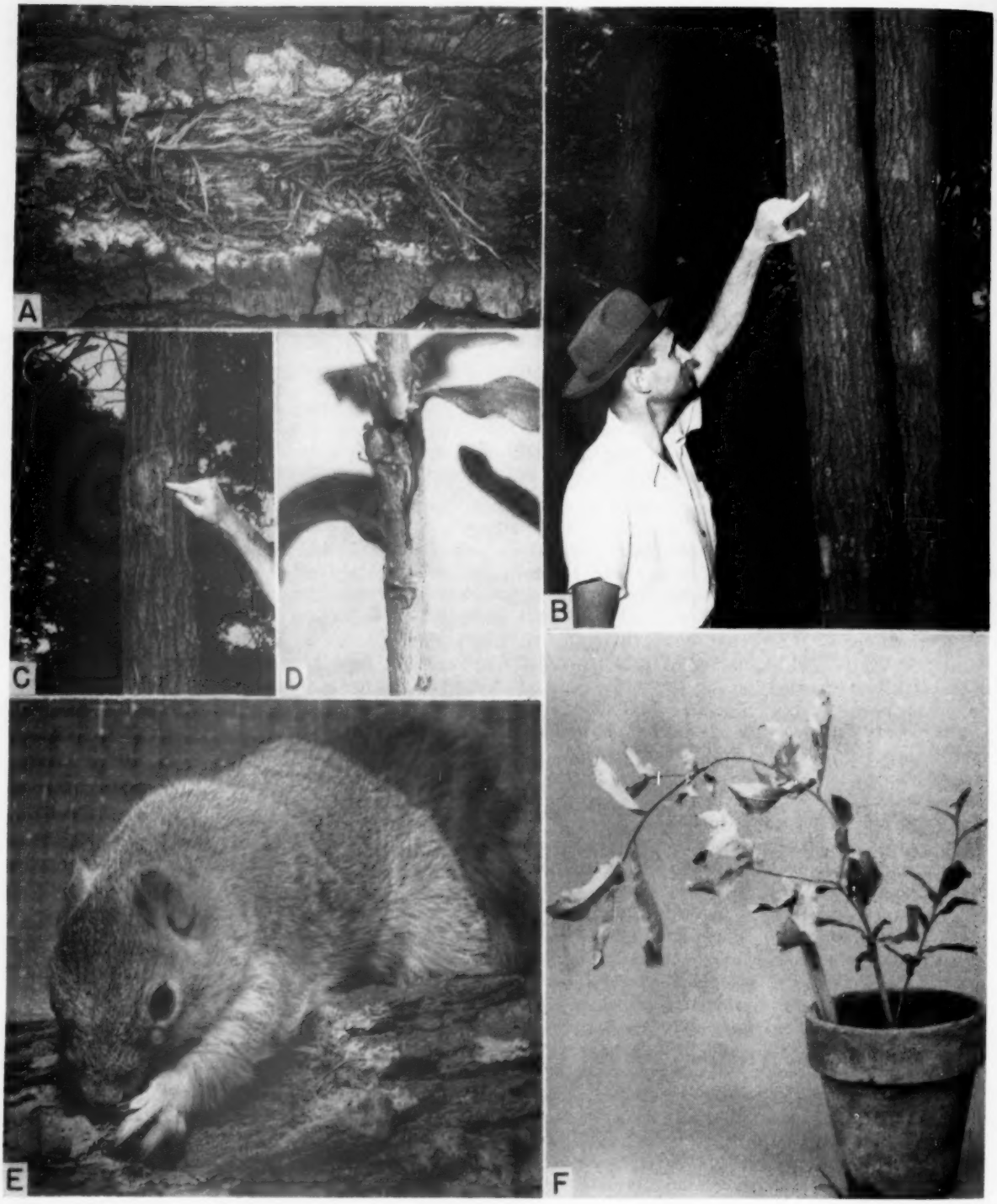

FIG. 1. A) A small shredded hole in the bark of a red oak caused by squirrel feeding that exposed the thick central pad of the oak wilt fungus mat. B) The trunks of 2 wilt-killed black oaks having numerous holes caused by squirrel feeding on the fungus mats. C) A large hole chewed by a squirrel in the bark of a black oak; the entire fungus mat is exposed. D) A typical wound on the stem of a potted live oak made by a fox squirrel. E) A caged squirrel eating portions of the exposed mat and pad after tearing off the oak bark. F) Typical oak wilt symptoms on a live oak that was wounded 31 days previously by a squirrel that had been fed fungus inoculum.

mycelium, and wheat bran. The suspension was then mixed with shelled corn and placed in 5 of the 6 cages. The squirrels readily ate the contaminated corn. One squirrel, caged with trees that served as controls, was fed corn only. Caged trees were examined daily, and those gnawed by the squirrels were removed and replaced with uninjured trees. Pots containing the gnawed trees were set in the ground and watered frequently.

Of 209 trees used in the experiment, 68 were gnawed by squirrels that had eaten food contaminated with the fungus, and 21 were gnawed by the squirrel in the control cage. The gnawing damage occurred during the months of March, April, May, and June. No trees were gnawed in July. Gnawing damage varied from localized bark removal to the clipping off of branches and buds. None of the damaged trees showed symptoms of oak wilt, and attempts to isolate the oak wilt fungus from them failed.

GREENHOUSE TRANSMISSION TESTS.—Further transmission tests were carried on in the greenhouse from October, 1953, through February, 1954. This experiment was designed to eliminate uncontrolled factors encountered outdoors such as high temperatures, deterioration of the fungus inoculum by contamination, and long intervals between the time when the squirrels ate the fungus and the time when they wounded healthy trees.

Test plants consisted of red oaks and live oaks (*Q. virginiana* Mill.), all of which were 2-year-old trees grown from acorns and transplanted in individual pots.

Conidia, conidia and ascospores, and fresh mats were used as inocula. Conidia were obtained from laboratory cultures of *E. fagacearum* grown from single-spored isolates on wheat-bran agar (75 g of wheat bran and 15 g of agar per liter of water) for 10-12 days at 24°C. For a source of ascospore-conidium inoculum, 2 compatible single-spored isolates were crossed on wheat-bran agar and grown for 12 days at 24°. Bolts that had been cut in January from a wilt-killed red oak and transported to the greenhouse provided the fresh mycelial mats. These bolts were used on the day of their arrival. Apparently only 1 strain of the fungus was present in the tree from which the bolts were taken as no perithecia developed on the mats.

All transmission work was done in a cool (21-24°C) basement room in the greenhouse. Three gray squirrels and 2 fox squirrels were each caged separately in cages that measured 3 × 3 × 3 ft. Each cage was equipped with a nesting box with a single entrance hole. To catch a squirrel, the animal was driven into a net placed over the entrance hole.

The inoculum containing conidia or conidia and ascospores was scraped from the surfaces of the cultures and smeared over slices of ripe apple. These slices were then placed in the cages, and the squirrels were watched until they had eaten them. After predetermined time periods, each squirrel was caught and held by 1 person while a second person held the stem of a tree near the mouth of the animal until it had wounded the stem near the base (Fig. 1, D). Usually, a squirrel was allowed to bite the stem only once. Each squirrel was induced or forced to wound 2–4 trees in succession before the animal was returned to its cage. One fox squirrel and 1 gray squirrel were used for each kind of inoculum. The fox squirrels wounded the trees of their own volition. The gray squirrels often had to be forced to make wounds. This was done by scraping the stem of a tree against the incisor teeth of the squirrel 1 or 2 times, thus producing a wound similar to that made by the fox squirrels. After the trees had been wounded, they were immediately transferred, for the remainder of the experimental period, from the basement room to the main greenhouse where the temperature ranged from 18–28°C. The interim between feeding on the conidium inoculum and wounding of the trees by the squirrels ranged from 20 minutes to 24 hours. Time intervals of 5 minutes to 24 hours were used when the squirrels were fed ascospore-conidium inoculum.

TABLE 1.—*Trees infected by the oak wilt fungus as a result of induced wounding by caged squirrels carrying inoculum on their mouth parts*

Interim between feeding on inoculum and wounding of trees by squirrels	Number of trees infected/Number of trees wounded for each type of inoculum[a]		
	Conidia	Ascospores and conidia	Mats and pads
5 mins.	----	2/2	----
15 mins.	----	2/3	----
20 mins.	1/3	2/4	2/3
30 mins.	1/3	6/7	2/9
45 mins.	2/3	0/7	0/2
1 hr.	0/5	7/8	2/4
1½ hrs.	0/5	1/6	
2 hrs.	3/5	4/5	
3 hrs.	0/6	2/3	
4 hrs.	3/7	2/6	
5 hrs.	1/6	0/6	
6 hrs.	6/6	0/6	
12 hrs.	3/6	0/6	
24 hrs.	0/6	0/6	
Total	20/61	28/75	6/18

[a] Controls: Number, 30; number infected, 0.

In tests with the third source of inoculum, oak bolts bearing fresh undisturbed mycelial mats were placed in individual cages with the animals. In each case a squirrel was watched until it had torn away the bark and had eaten portions of a mat and central pad (Fig. 1, E). It was then caught and allowed or forced to wound trees in the manner described above. The intervals of time that elapsed between the time a squirrel fed on a mat and the time it was caused to wound the trees ranged from 20 minutes to 1 hour. Because of the limited source of fresh mycelial mats at the time of the experimental transmission work, longer time intervals were not attempted.

Thirty trees wounded by a fox squirrel, which had been fed noninoculated apple, served as controls. All trees were observed closely and those developing foliar

symptoms of oak wilt (Fig. 1, F) were cultured for the oak wilt fungus. The oak wilt fungus was successfully isolated from 54 trees. The time required for the trees to show typical oak wilt symptoms after they had been wounded by the squirrels ranged from 20 to 81 days regardless of the source of inoculum. None of the trees used as controls wilted. The data presented in Table 1 include the combined results for fox and gray squirrels, as the separate results for the 2 species showed no apparent differences.

Several materials were tested for their ability to discourage squirrel-feeding on mycelial mats.[2] Bolts cut from mat-producing trees were sprayed with various materials and then placed in the cages with the squirrels. The tested materials that failed to prevent the squirrels from chewing off the bark and eating the fungus were zinc dimethyldithiocarbamate (ziram), kerosene, mineral seal oil, engine oil (No. 10 weight), lime sulfur+$CaCO_3$+$CuSO_4$, turpentine, turpentine+tar (roofing), kerosene+tar (roofing), and penta-chlorophenol. The only material that prevented the squirrels from gnawing the bark and eating the fungus was creosote used either in full strength or diluted to ¾ strength with kerosene.

Discussion.—Rodent damage to healthy oaks occurs commonly in forests of Illinois. This damage includes clipping of twigs and buds, gnawing wounds on large and small branches, and gnawing wounds around knots and callused branch stubs. Most of the gnawing damage occurs during the winter and spring when there is considerable feeding by rodents on mycelial mats of the oak wilt fungus.

Himelick et al (4) earlier pointed out several factors that implicate squirrels in such feeding. Further, in the present studies, the tooth-mark sizes and patterns made by the gnawing on oak bolts by fox and gray squirrels in captivity corresponded closely to the sizes and patterns of tooth-marks made by squirrels in nature. Holes gnawed by squirrels in the bark of mat-bearing oak bolts in the greenhouse were identical to those observed many times on mat-bearing oaks in the field. The readiness with which the squirrels in captivity sought and ate the fungus pads or pressure cushions on oak bolts in the greenhouse leaves little doubt that these animals also eat the fungus in the field. The tests described in this paper show that squirrels can carry viable inoculum on their mouth parts for at least 12 hours after they have fed on the fungus. Presumably, they can transmit the fungus to healthy oaks, which they gnaw on in the field, within that period of time. Either conidia or ascospores may be important in transmission of the fungus by squirrels. Other possible forms of inoculum might be the fungus mycelium or the cells that comprise the fungus pads.

Surveys made in 1953 and 1954 showed a pronounced increase in the number of mat-producing trees damaged, in the number of holes per tree, and in the average size of the holes that were gnawed. It is interesting to note that these observations are similar in part to findings reported by Mielke (5) in a review dealing with rodent feeding on white pine blister rust cankers. Both Clark (2) and Mielke (5) reported that squirrels were slow to learn of the tasty rust canker, but once the new source of food was discovered, feeding on the cankers generally increased each year. Mielke further pointed out that, in the eastern states, most of the feeding on blister rust cankers by rodents took place in late winter and early spring. This also coincides with our observations in Illinois concerning the seasons of most active feeding by the squirrels on the oak wilt fungus.

If rodent feeding on the fruiting mats of *E. fagacearum* continues to increase at the present rate in the forests of Illinois and the climatic conditions are favorable, these animals might conceivably become important vectors in the local spread of the oak wilt disease. Furthermore, their intensive feeding on the fungus mats might reduce the significance of insects as possible vectors. Exposure of the mats by squirrel feeding results in an early deterioration of the fungus inoculum by drying and contamination.

While it does not seem advisable at present to attempt to limit the squirrel population in oak wilt areas, creosote used as a repellent appears to discourage squirrels from chewing off the bark and feeding on the mycelial mats.

[2] This portion of the investigation was carried on in cooperation with Dr. Paul Hoffman formerly with the Illinois Natural History Survey.

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A COMPARATIVE STUDY OF SPORE GERMINATION OF USTILAGO TRITICI AND U. NUDA [1]

W. Popp

SUMMARY

Observations were made on spore germination in 5 races of *Ustilago tritici* and 6 races of *U. nuda* on potato-dextrose agar and water agar. On potato-dextrose agar, chlamydospores of *U. tritici* produce relatively long promycelia, individual cells of which develop into branched hyphae of monocaryotic cells. As a result of continuous budding, the cells of the branches simulate sporidia but remain attached to each other. Later, the monocaryotic cells conjugate in pairs and initiate dicaryotic growth. Spores of *U. nuda* on potato-dextrose agar produce promycelia about half as long as those of *U. tritici*. The compatible cells of a promycelium of *U. nuda* conjugate in pairs to give rise to binucleate hyphal branches, each of which subdivides usually into 1 dicaryotic and 2 monocaryotic cells. The dicaryotic cells branch and the monocaryotic cells conjugate to give rise again to dicaryotic hyphae, each of which subdivides into 1 dicaryotic and 2 monocaryotic cells. The growth process is therefore alternately dicaryotic and monocaryotic.

On water agar, germination in both species of smut fungi is similar in that promycelial cells conjugate in pairs, and each pair gives rise to a dicaryotic infection hypha. There is, however, a slight tendency of some promycelial cells of *U. tritici* to sprout and divide before fusion with a compatible cell.

The 2 smuts differ fundamentally in their type of spore germination, thus providing a morphological basis for their retention as 2 distinct species.

In germination tests of chlamydospores of *Ustilago tritici* (Pers.) Rostr. and of *U. nuda* (Jens.) Rostr., the type of germination produced on potato-dextrose agar by many different collections has been observed. Deviation from published characterizations of germination that both smut fungi are supposed to possess in common was conspicuous, and it appeared that they were not identical in certain structural details.

The 2 smuts generally have been considered identical in all respects except host specialization (1, 3, 4, 5, 6, 7, 10), but marked differences in cultural characters have been reported by Thren (12). The current concept of spore germination for *U. tritici* and *U. nuda* probably is based on results obtained with spores germinating in water or nutrient solutions (8, 11). These descriptions become inadequate as new methods of inducing spore germination and improved methods of examination make possible more detailed observations. Comparative experiments, therefore, were undertaken to determine nuclear and structural characteristics produced on solid media by germinating spores of *U. tritici* and of *U. nuda*.

MATERIALS AND METHODS.—Chlamydospores of 5 different races of *U. tritici* and 6 of *U. nuda* from various sources in Canada were germinated on Difco potato-dextrose agar and on water agar in Petri dishes. An effort was made to examine the fungi in different stages of spore germination during an incubation period of 24 hours at temperatures ranging from 19 to 21°C. To secure contact with the agar and uniformity in germination, the chlamydospores were suspended in a drop of sterilized distilled water and then spread over the agar surface with a sterilized squirrel-tail brush. In the preparation of the Difco potato-dextrose agar, Bacto agar was added to the medium to raise the agar content to 2 per cent. This gave a firm surface that facilitated the distribution of the spores evenly over the agar. Contamination of the cultures by bacteria usually was not a problem, as most of the smut was collected from plants grown in the greenhouse. When spores from field collections were germinated, however, the agar had to be acidified to ensure control of bacterial contamination. Detection of structural details was facilitated by cutting out a square piece of agar, placing it on a microscope slide, and flooding the germinating spores with a drop of staining solution[2] after the free water on the surface of the agar had evaporated. A cover glass was placed on top of the preparation, and examination was made under the high dry power of the microscope. The procedure resulted in staining the protoplasmic contents but not the septa, which remained hyaline. All structures, therefore, were made easily detectable. Figure 1, A is a photomicrograph of 2 spores of *U. tritici* and 2 spores of *U. nuda* germinating on potato-dextrose agar.

To determine the nuclear condition of germinating spores, the crystal-violet staining procedure described by Oehlkers (9) was followed. The material was first killed and fixed in Belling's modified Navashin fluid, then affixed to glass slides smeared thinly with Haupt's adhesive.

Measurements of promycelial lengths were made only after the germinating spores had been stained with cotton blue in lactophenol and had been mounted

[1] Accepted for publication June 25, 1955.
Contribution No. 1463 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

[2] Lactic acid, 20 ml; phenol, 20 ml; glycerol, 10 ml; distilled water, 20 ml; and Poirrer's blue (cotton blue), 0.5 per cent.

by the method previously described. Only promycelia having septa were measured because the presence of the latter was considered to be an indication of full development in length. Lengths reported are means and ranges of at least 100 measurements.

RESULTS.—*Germination on Difco potato-dextrose agar.*—Camera lucida drawings of spores of *U. tritici* and *U. nuda* in different stages of germination on potato-dextrose agar are reproduced in Figure 2, A, B. A schematic drawing of nuclear behavior during successive stages of germination of spores of *U. nuda* on potato-dextrose agar is shown in Figure 3.

Promycelia of chlamydospores of *U. tritici* (Fig. 2, A) are relatively long and become subdivided first into 2 and then into 4 cells, following the first and second meiotic divisions, respectively. Individual cells of the promycelium then sprout or bud, their nuclei divide, and a septum forms between the daughter nuclei.

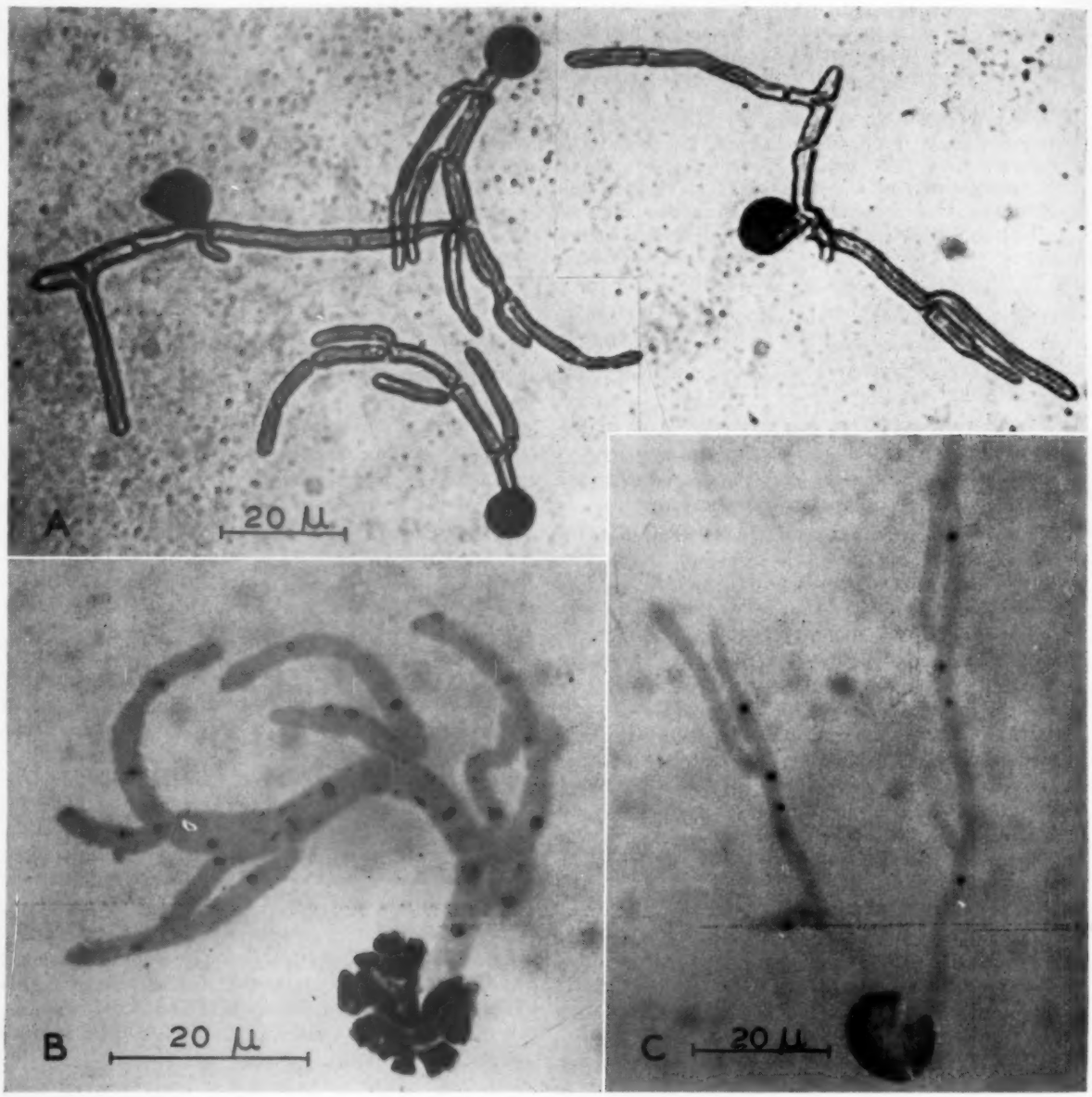

FIG. 1. A) Two germinating chlamydospores of *Ustilago tritici* (center) and 2 of *U. nuda* (right and left) showing growth developing from individual cells (*U. tritici*) and from conjugate cells (*U. nuda*) of the promycelium. Spores were germinated on the same plate of potato-dextrose agar and photographed after being stained with cotton blue in lactophenol. B and C) Photomicrographs of stained preparations of a spore of *U. tritici* (B) and 1 of *U. nuda* (C), showing monocaryotic condition of the promycelial branches of *U. tritici* and the paired and single nuclei in each of the 2 hyphae produced by the promycelium of *U. nuda*.

This septum may be laid down in the promycelium or it may form outside of it in the young developing branch. As a result, some promycelia are more than 4-celled after branching. Subsequent growth involves the continuous sprouting or budding of cells of the promycelial branches and their division, by means of septa, into daughter cells. The cells are monocaryotic (Fig. 1, B) and simulate sporidia in their development, but remain attached and form a complicated system of branching. Several branches may arise from

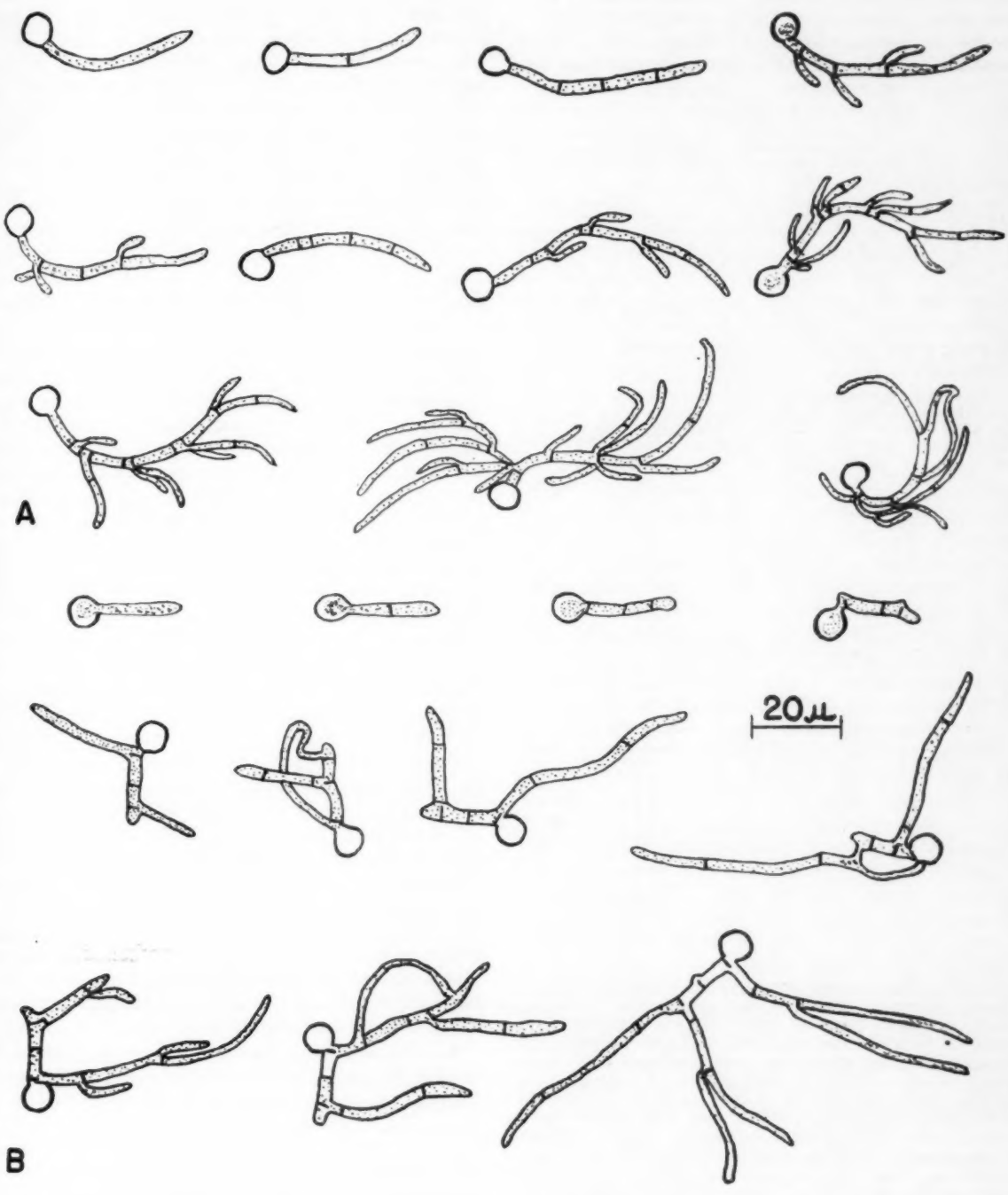

FIG. 2. Camera lucida drawings of germinating spores in different stages of development on potato-dextrose agar. A) *U. tritici*. B) *U. nuda*.

a promycelial cell. After considerable branching has taken place, compatible cells fuse in pairs. This fusion apparently initiates dicaryotic growth as described by Thren (12).

Promycelia of spores of *U. nuda* (Fig. 2, B), as compared to those of *U. tritici*, are relatively short and are 3- to 4-celled after meiosis. The chlamydospore often acts as the fourth cell. Immediately after their formation, the promycelial cells conjugate in pairs by means of conjugation tubes between adjacent or nonadjacent cells. Where spores germinate near one another, a cell of one promycelium sometimes fuses with that of another. Each fused pair of cells gives rise to a dicaryotic hypha (Fig. 3, A, B) that may develop either from the conjugation tube or from the end of one of the fused pair of cells. After the hyphae have developed to a certain length, division of the conjugate nuclei takes place (Fig. 3, C) and septa are laid down, subdividing each hypha into 1 dicaryotic and 2 monocaryotic cells (Fig. 1, C, 3, D). The promycelial cells often remain filled or partly filled with protoplasm and form the basal portion of the hyphae that develop into a branching mycelium. In this process, the compatible cells of a promycelium conjugate in pairs to produce binucleate hyphal branches, each of which subdivides, usually into 1 dicaryotic and 2 monocaryotic cells. The dicaryotic cells branch, and the monocaryotic cells conjugate to give rise again to dicaryotic hyphae, each of which subdivides into 1 dicaryotic and 2 monocaryotic cells (Fig. 3, E-G). This type of growth is similar to that described by Thren (12) for dicaryotic cultures of *U. nuda*.

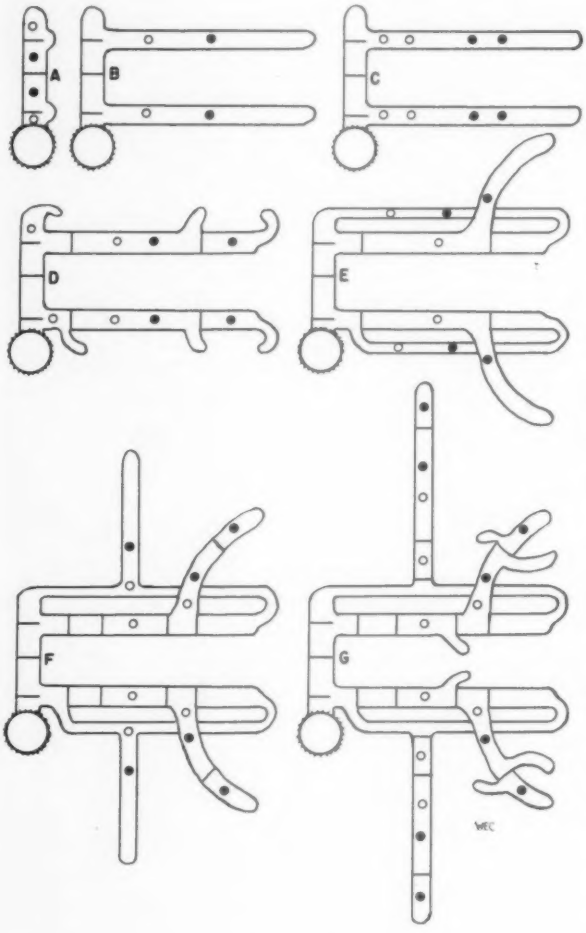

FIG. 3. Diagrammatic representation of nuclear behavior during progressive stages of spore germination of *Ustilago nuda* on potato-dextrose agar. A) Monocaryotic (compatible) cells of the promycelium fused in pairs. B) dicaryotic hyphae developing from conjugate cells of the promycelium. C) Distribution of daughter nuclei in the hyphae following division of the conjugate nuclei (B). D) Subdivision of each hypha into a dicaryotic and 2 monocaryotic cells by means of septa. Note conjugation tubes forming from monocaryotic cells and the branch developing from each dicaryotic cell. E-G) Conjugation of the monocaryotic cells and branching of the dicaryotic cell of each hypha to give rise again to dicaryotic hyphae, each of which subdivides after division of the conjugate nuclei into 1 dicaryotic and 2 monocaryotic cells.

Germination on water agar.—On water agar, spores of *U. tritici* produce a 4-celled promycelium that sometimes grows out for a short distance beyond the limits of the spore wall but remains connected to the spore by the older, empty portion which the protoplasm has evacuated. Cells of the promycelium produce conjugation tubes and fuse in pairs, although there sometimes is a tendency for an individual cell to branch and undergo nuclear and cell division once or twice before conjugation. Each fused pair of cells gives rise to a binucleate (infection) hypha developing either from the conjugation tube or from one of the fused pair of cells. These hyphae grow out in "Suchfaden" fashion, growth forming at the tip, whereas the older basal portions become progressively empty and septate in the wake of the evacuating protoplasm.

Promycelia of spores of *U. nuda* are 3- to 4-celled on water agar, the chlamydospore often functioning as the fourth cell. The cells usually conjugate in pairs immediately following their formation. Each pair of cells produces a binucleate (infection) hypha. These hyphae grow along the surface of the agar and leave older portions empty and septate as a result of evacuation of the protoplasm. On water agar, germination of spores of *U. nuda*, therefore, is similar to that described for *U. tritici* except for the tendency of some promycelia of the latter to prolong the monocaryotic condition after meiosis.

Lengths of promycelia.—In the course of the study, camera lucida drawings of the germinating spores usually revealed a distinct difference between *U. tritici* and *U. nuda* in promycelial length. A detailed study of this characteristic, therefore, was made. Chlamydospores were germinated on potato-dextrose agar and on water agar. In the measurement of the promycelia of *U. tritici*, the old or basal portion which the protoplasm had evacuated was not included in

TABLE 1.—*Lengths of promycelia of germinating chlamydospores of races of Ustilago tritici and U. nuda, produced on potato-dextrose agar and water agar*

Species and race	Length of promycelia (μ) Potato-dextrose agar Range	Mean	Water agar Range	Mean
U. tritici				
Race R1	25-54	39.5	25-52	38.5
Race R2	22-59	39.0	23-59	41.0
Race R3	23-66	44.5	24-59	41.5
Race R4	22-58	40.0	22-54	38.0
Race 50-8	22-53	37.5	24-52	38.0
All races	22-66	40.1	22-59	39.4
U. nuda				
Race R1	13-25	19.0	13-24	19.5
Race R2	13-24	18.5	13-22	17.5
Race 49-70	13-24	18.5	12-29	20.5
Race 49-68	13-25	19.0	13-28	20.5
Race 47-14	15-21	18.0	13-25	19.0
Race 51-20	13-21	17.0	12-24	18.0
All races	13-25	18.3	12-29	19.1

measurements, since it was no longer a part of the active structure.

Both on water agar and on potato-dextrose agar, the mean length of promycelia of spores of *U. tritici* are approximately twice that of spores of *U. nuda* (Table 1). Individual promycelia of the 2 smut fungi sometimes approximate each other in length, but a wide difference in average length is usually observed after only a few promycelia selected at random in each fungus have been measured. A slight variation in promycelial length exists among the races of each fungus, but this does not mask the difference between the species.

Exceptionally long promycelia, ranging from 75 to 90 μ in length, were encountered among germinating spores of *U. tritici*. The data in Table 1 do not include such lengths, however, because of their infrequent occurrence. The determination of their significance is being left for future study.

DISCUSSION.—*Ustilago tritici* and *U. nuda* have long been separated as distinct species on a host specialization basis (2). Cunningham (3) and Rodenhiser (10) expressed the view that the 2 smut fungi are not distinct species but are specialized races of the same species since they are similar in morphology and differ only in their pathogenicity. Acting on the same principle, Fischer and Hirschorn (6), Ainsworth and Sampson (1), and Fischer (5) used morphological characters in place of host specialization as a means of species separation, and even went so far as to combine *U. tritici* and *U. nuda* under the name *U. nuda*, because of the mycelial type of germination, and to combine *U. avenae* (Pers.) Rostr. and *U. nigra* Tapke under the name *U. avenae*, because of the sporidial type.

Thren (12), on the contrary, found that in culture, spores of *U. nuda* produce a more stable dicaryotic growth than do spores of *U. tritici* and that dicaryotic colonies of the 2 fungi are easily distinguishable under the microscope. He concluded that the 2 fungi are different species and not specialized races or varieties of the same species.

The writer considers that when the 2 fungi, *U. tritici* and *U. nuda*, are produced on potato-dextrose agar, there are good morphological distinctions between the 2, in structure and manner of production of the promycelial outgrowths. *U. tritici* produces growth only from individual cells of the promycelium, whereas *U. nuda* develops it only from conjugate cells. The 2 smut fungi, therefore, may be readily identified by this striking difference (Fig. 1, A). Furthermore, identification is considered convenient and practicable enough for use in taxonomy, particularly with the method of examination used in these studies.

The difference in promycelial length of the 2 fungi is a wide one and is an additional differentiating characteristic. When produced either on water agar or potato-dextrose agar, promycelia of *U. tritici* are about twice those of *U. nuda* in mean length. Statistical calculations are unnecessary as a wide difference in mean length is apparent after only a few promycelia are measured.

Another differentiating structural character, although not suitable for quick diagnosis, is that of the nuclear condition of promycelial branches produced on potato-dextrose agar (Fig. 1, B, C). The promycelial branches of *U. tritici* are monocaryotic, whereas those of *U. nuda* are predominantly dicaryotic (Fig. 3).

The experimental work, therefore, has shown that *U. tritici* and *U. nuda* are not identical in type of spore germination. Instead, *U. tritici* apparently resembles the sporidial smut fungi, *U. avenae* and *U. nigra*, since outgrowths of the promycelium of these fungi originate from individual cells and are the result of a continuous process of budding and cell division. There is a difference, however, in that in *U. tritici* new cell growth is cut off by means of a septum and remains attached, whereas in the sporidial smuts it is budded off in a yeast-like manner. In contrast, the spore germination of *U. nuda* is unique in that growth is mycelial and develops from conjugate cells of the promycelium. Thus, 3 types of spore germination, in place of the 2 types (sporidial and mycelial) used in Fischer's scheme of classification (6), are recognizable among *U. tritici*, *U. nuda*, *U. avenae*, and *U. nigra*.

The conclusion is that *U. tritici* and *U. nuda* are separable at the species level whether the scheme of classification is based on host specialization (2) or on morphology of germinating spores (5, 7). If separation based on host specialization is adhered to, the results of this investigation serve no useful purpose except as a morphological or a quick method of identification. If that based on characteristics of germinating spores is adopted (1, 4, 5, 6), a change in definition and in recognition of the 2 smut fungi as distinct species is warranted. These results also necessitate changes in the published characterizations of spore germination of the 2 fungi.

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EFFECTS OF SEA WATER SPRAYS ON PINEAPPLE PLANTS[1]

C. P. Sideris

SUMMARY

Pineapple plants grown in fields located within 1.5 miles from the sea and exposed to wind-blown sea water sprays develop leaf-tip necrosis that extends toward the proximal tissues and may result in death of approximately 25 per cent of the leaf area. The symptoms of the injury appear as bands of alternating dark-brown and light-brown hues. Leaf tissue injury is more extensive in old and mature leaves than in young ones, because of chloride intake for longer periods by the former than by the latter. Salt deposition and severity of leaf-tip necrosis were inversely proportional to the distance from the sea. Chloride solutions applied to pineapple plants grown in fields beyond the range of wind-blown sea water sprays produced symptoms of leaf-tip necrosis and materially reduced the yields and quality of the fruits. The injury was found to be caused by Cl and not by Na.

Partial control of the chloride injury is effected by plantings in multiple rows of *Casuarina equisetifolia* on the seaward side of pineapple fields for the trapping of wind-blown sea water. Soil fumigation, presumably because of better nutrition resulting from elimination of organisms harmful to the roots, did not reduce materially leaf-tip necrosis but increased plant growth with superior fruit weights and quality, thus overcoming the adverse effects of chlorides on fruit yield. Some evidence was obtained that other treatments that stimulate vigorous plant growth, such as ample applications of nitrogen and potassium, will reduce the adverse effects of chlorides on yield and quality of fruits.

Ananas comosus (L.) Merr., when grown in certain fields on the islands of Kauai and Maui in Hawaii, is subjected to wind-blown sea water sprays that cause injuries ranging from 10 to 80 per cent of the area of the old and mature leaves. The injured areas, of soft or dry dead tissues, show successive zones of alternating dark brown and light brown hues (Fig. 1). The young leaves usually show less salt injury than do the older leaves (20).

Boyce (6) ably presented the literature on the effects of sea water sprays on plants in "the salt spray community". Boyce (4, 5), Oosting and Billings (14), and Oosting (13) have shown that plants respond variously to salt water sprays, some undergoing hypertrophy and others developing injury. Wells (23) and Wells and Shunk (24) attributed the predominence of *Quercus virginiana* in coastal areas of the Atlantic to the tolerance of its mature leaves to salt sprays. Harter (10) observed that the leaves of wheat, oats, and barley grown in soils treated with sodium chloride at concentrations of 0.7, 1.0, and 1.4 per cent decreased moisture losses by transpiration by developing a pronounced waxy bloom, a cuticle 2.6–3.2 μ thick, and small epidermal cells. Toxic effects of chlorides were observed on a variety of agricultural food plants by Eaton (7), Tottingham (22), and Harvey and

[1] Accepted for publication June 30, 1955.
Published with the approval of the Director as Technical Paper No. 233 of the Pineapple Research Institute of Honolulu, Hawaii.

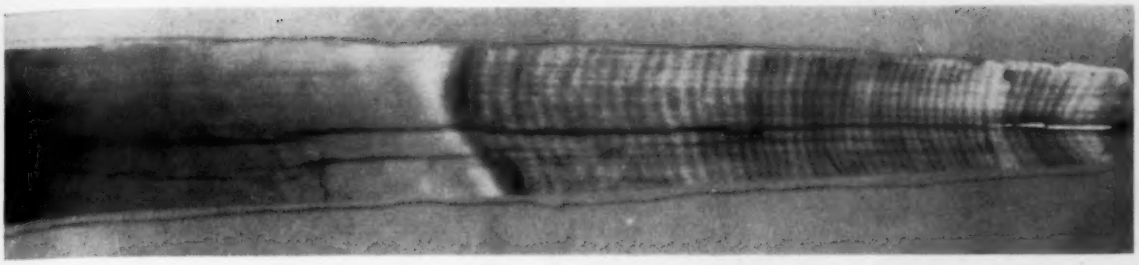

FIG. 1. Symptoms in pineapple leaf of leaf-tip necrosis caused by high Cl concentrations.

Toth (11). Haas (8, 9) and Ayers and associates (2, 3) observed leaf-tip burn of Fuerte avocado at Cl concentrations greater than 0.5 per cent. Nightingale and Yoshida(12) state that certain varieties of soybeans showed greater tolerance to furrow than to sprinkler applications of water containing 96 grains of sodium chloride, while sorghum varieties tolerated both types of application equally well.

The studies reported herein pertain to the amounts of sodium chloride in sea water sprays blown inland by winds and deposited in pineapple fields, rain in relation to leaf injury caused by chlorides, and harmful effects of chloride salts when applied to plants in combination with various fertilizer treatments.

METHODS AND MATERIALS.—The amounts of wind-blown sea water in pineapple fields on Kauai and Maui were measured by the total content of Cl in the water collected in rain gauges and in adjacent halatometers. The halatometer is an instrument for the trapping and storing of air-borne particles and water droplets that come in contact with the metal sides of an oblique funnel-shaped opening (16). The instrument is rotated by a vane, so that the opening always is in the prevailing wind. The contents of the receptacles in the halatometers and rain gauges were collected at monthly intervals, except during seasons of very heavy precipitation, and measured volumetrically. Cl was determined by Patterson's method, as modified by Hald (15). Determinations of fruit quality were based on Brix (specific gravity of expressed juice), on acidity as percentage of critic acid (normal acidity of expressed juice $\times 0.064 \times 100$), and on tissue translucence (percentage of the cut surface that is translucent when fruits are bisected lengthwise). Sugars were estimated from Brix by the method of Abel et al (1).

Leaves were divided into the following groups: old, mature, active, and young (18). Approximate values for the surface area and weight of the leaves of these groups, calculated as percentage of the entire leaf system of 1-year-old plants were 10, 40, 35, and 15, for the old, mature, active, and young groups, respectively. The relative amount of leaf-tip necrosis in separate leaf groups was calculated by dividing the length of an entire leaf by the length of the discolored area and multiplying by 100. Since pineapple leaves are tapered, this gives only an approximation of the amounts of necrosis. Data calculated by this method, however, should be relative. The amount of leaf necrosis for an entire plant was calculated as the sum of the weighted leaf-group.

Leaf-tip necrosis was studied in pineapple fields on the islands of Kauai, Maui, and Oahu (Wahiawa). The fields on Kauai and Maui were within 1.5 miles of the sea and were exposed to wind-blown sea water sprays. The field at Wahiawa (on Oahu) approximately 15 miles away from the sea and beyond the range of wind-blown sea water sprays, was employed for testing chlorides applied to pineapple plants in combination with various nutrient salts. Also, data were obtained in the laboratory on the intake of chlorides by intact pineapple leaves.

RESULTS.—*Rainfall, salt deposition, and leaf-tip necrosis in fields at various distances from the sea.*—1) *Kauai.*—The fields 7-A, 5, and Camp 1, in which the experiment was located, are exposed to northerly, easterly, and north-easterly winds, but are protected from southerly and westerly winds by a range of mountains rising abruptly at the southern edge of the fields. Some miles to the west the main mountain range reaches the ocean, preventing strong wind currents from that direction.

Field 7-A, the closest to the sea, is relatively level, with slow drainage and leaching of salt deposits. It is exposed to more frequent salt sprays than the other fields. Field 5, located on a hill and further away from the sea, is exposed to sea water sprays, mostly on the seaward side. Camp 1, the furthest from the sea and located on level land, is exposed to sea water sprays only at times of extremely strong wind currents. Records taken during the period from May 1944 to May 1946 show that water deposits correlated directly and salt deposits inversely with the distances of the fields from the sea (Table 1). The coefficient of variation of salt deposits in these fields increased as the distance from the sea increased, because of greater fluctuations in the movement of salt spray by wind currents at long than at short distances from the sea.

In 1942, the monthly deposition of water and salt in the halatometer in field 7-A varied greatly (Fig. 2, A, B). In June, the deposits of salt exceeded 900 lb. per acre, and the water deposits were less than 1 in. These salt deposits in June resulted from high wind velocities and were 4.5 times as great in the halatometer as in the rain gauge. Water deposits were

TABLE 1.—*Deposits of salt and rain-water on fields located at various distances from the sea*

Periods	Field 7-A 1050 ft. to sea Salt	Water	Field 5 4500 ft. to sea Salt	Water	Camp 1 7050 ft. to sea Salt	Water
	lb./acre	in.	lb./acre	in.	lb./acre	in.
1944, May-Dec.	102	14.9	61	15	41	16.6
1945, Jan.-June	60	21.8	56	19.5	27	25.5
1945, July-Dec.	64	19.8	46	19.9	37	22.8
1946, Jan.-May	106	23.5	72	26.9	68	31.4
Monthly means	13.8	3.3	9	3.4	7.2	4.0
Standard deviation	9.5		6.7		6.2	
Coefficient of variation	69		74		85	

about the same in the 2 instruments. Leaf-tip necrosis, which ensued following the great salt deposits in June, was estimated as amounting to 21.8 per cent in August (Table 2).

In field 5, leaf-tip necrosis, amounting to approximately 7.8 per cent, was mostly confined to the plants on the seaward side of the hill. In this field, the plants grown at the base of the hill in a basin-like area of a few acres had as much leaf-tip necrosis as those in Field 7-A. Following the construction of a subterranean drainage outlet in this area, however, leaf-tip necrosis has almost disappeared.

Salt deposition was lowest in Camp 1, and the incidence of leaf-tip necrosis was so low that it had no adverse effects on subsequent plant growth.

TABLE 2.—*Approximate percentage of discolored leaf areas in different groups of leaves of approximately 1-year-old pineapple plants in various fields*

Field	Length of discolored area / Length of entire leaf × 100[a] Old leaves (10)[b]	Mature leaves (40)[b]	Active leaves (35)[b]	Young leaves (15)[b]	All leaves
Kauai, 7-A	57.1	27.3	12.5	5.6	20.5
Kauai, 5	31.3	8.3	3.8	0.0	7.7
Kauai, Camp 1	18.8	4.2	0.0	0.0	3.6
Maui, B	62.5	32.0	14.8	5.3	25.1
Wahiawa					
Treatment 2	47.0	16.7	6.3	0.0	13.6
Treatment 3	62.5	32.0	14.8	5.6	25.0
Treatment 10	80.0	45.0	20.0	7.5	37.6

[a] Approximately 10 plants, each with 40–60 leaves, were taken from each field and examined for leaf-tip discoloration. Except for the Wahiawa field, all fields were within the range of wind-blown sea water.

[b] Figures in parentheses indicate the percentage of all leaves that were classed as indicated.

It is evident from the results (Table 2), that leaf-tip necrosis is more extensive in old and mature leaves than in young ones, presumably because of longer intervals of chloride intake by the older leaves. Also, aging may increase the susceptibility of leaves to necrosis.

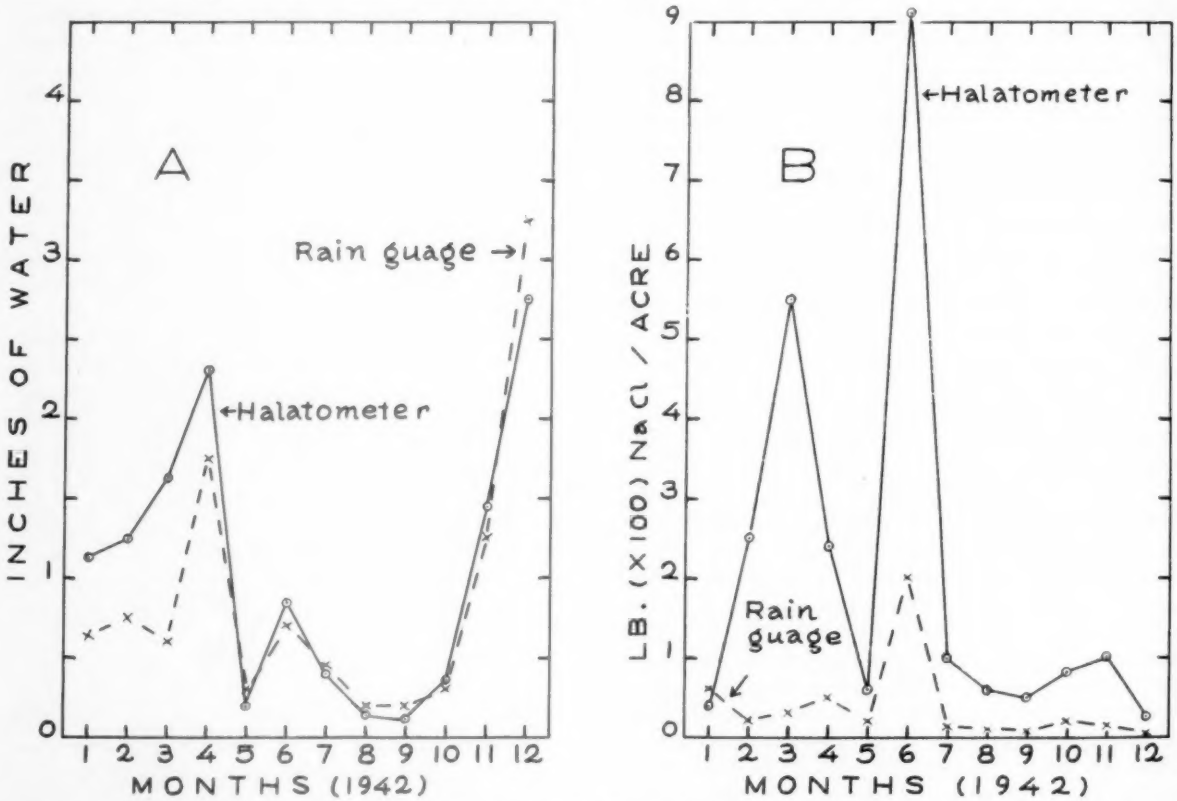

FIG. 2. Monthly deposits in halatometer and rain gauge in Maui during 1942. A) Rain water. B) Sodium chloride.

TABLE 3.—*Quarterly deposits of salt and water per halatometer in Field B on Maui and Field 7-A on Kauai during 1942*

Periods	Maui (B) 450 ft. to sea		Kauai (7-A) 1050 ft. to sea	
	Salt	Water	Salt	Water
	g	l	g	l
January-March	21.0	43.3	11.9	55.7
April-June	29.4	38.2	18.0	46.7
July-September	6.4	48.3	3.1	10.3
October-December	2.9	27.7	2.8	64.7
Total	59.7	157.5	35.8	177.4

2) *Maui.*—Field B of the Maui Pineapple Company, located at a distance of 450 ft. from the sea and at an elevation of 175 ft., has a slope ranging in various areas from 5 to 15 per cent. It is exposed to northerly, and north-easterly winds. Quarterly salt deposits in the halatometer (Table 3) varied throughout the year; they were greater in the April-June quarter than in the others. Likewise, the intensity of leaf-tip necrosis, assessed in August, 1942, was greater for the April-June quarter than for the others. Plants in the areas exposed to wind currents from the sea showed more necrosis than areas sheltered from the wind.

Leaf-tip necrosis was more prevalent in the Maui than in the Kauai 7-A field (Table 2). Also, the quarterly deposits of salt were greater and water deposits smaller in the Maui than for the Kauai fields (Tables 1 and 3). Estimated salt concentrations in the combined aqueous solutions of the halatometers deposited in the April–June quarter, averaged 0.77 and 0.39 per cent, respectively, for the Maui and Kauai 7-A field.

Effect of adding Cl in combination with nutrients.—In Field 3-A at the Wahiawa substation, which is beyond the range of wind-blown sea water spray, different amounts of Cl in combination with N, P, K, Ca, and Na were applied to the plants. Cl was added as KCl and $CaCl_2$ and Na as Na_2SO_4 in order to separate the responses to Cl and Na. At 6 bimonthly intervals solutions were poured by hand into the axils of the leaves at the center of the plant. The soil on ½ of each plot was fumigated with chloropicrin (trichloronitromethane) at the rate of 150 lb. per acre.

The data indicate that Cl at 990 lb. per acre increased leaf-tip necrosis (Table 2) but decreased fruit weight and tissue translucency (Table 4). There was indication that such changes were modified by K and by N, which stimulated vigorous plant growth. Na at 600 lb. per acre did not cause any adverse effects, indicating that the harmful effects of NaCl are due to Cl.

Fumigation of the soil resulted in an increase in fruit weight and in translucency of the fruit (Table 4). Approximate percentages of total leaf-tip necrosis, calculated as in Table 2, were approximately the same on nonfumigated plots and on otherwise comparable fumigated plots. The favorable effects of soil fumigation resulted from the development of larger plants with fruits of better quality than without fumigation. These beneficial effects of fumigation were attributed to the better nutrition that resulted from the elimination of organisms harmful to the roots (19, 21).

Cl content of leaves.—The Cl content of discolored and dead tissues of necrotic leaves of plants exposed to sea water sprays varied from 3.5 to 4.5 per cent of dry weight. In plants grown in nutrient solutions with 280 ppm of Cl and 3.9 ppm of K, the Cl content of necrotic leaf areas ranged from 1.0 to 1.5 per cent of dry weight (19). These results suggest that the Cl content of the tissues is not at all times a satisfactory index of Cl toxicity. This is consistent with the observations that nutritional factors alter the extent of plant tolerance to Cl.

Intake of Cl by leaves.—In plants grown at a distance from the sea, all Cl intake is effected by the roots. In plants grown near the sea and exposed to frequent sea water sprays, salt is deposited on the leaves. In an experiment simulating the latter conditions, evidence was obtained indicating that Cl intake

TABLE 4.—*Properties of fruits harvested from plants grown in fumigated and nonfumigated soil in field 3-A at Wahiawa and supplied with various elements in solution except $CaSO_4$ which was applied to the axils of old leaves as a solid*[a]

Treatments	Elements, lb. per acre of 16,000 plants			Fruit weight lb. Plant crop			Brix %		Citric acid %		Translucence %	
	Cl[b]	K[c]	N[d]	Fumigated	Not fumigated	Ratoon	Fumigated	Not fumigated	Fumigated	Not fumigated	Fumigated	Not fumigated
1	990	1110	800	6.09	4.31	3.42	16.9	16.2	0.60	0.77	98	36
2	99	1110	800	5.59	5.57	4.13	17.4	16.2	0.62	0.71	100	93
3	990	111	800	5.33	4.66	2.46	16.8	15.6	0.67	0.79	98	53
4	500	555	800	5.26	4.78	3.98	17.3	15.6	0.77	0.76	93	84
5	99	1110	400	5.91	5.67	4.23	16.3	16.6	0.73	0.85	100	91
6	990	111	400	5.47	4.47	2.86	17.0	16.0	0.75	0.78	83	57
7	500	555	400	4.93	4.81	3.73	17.2	15.9	0.82	0.85	79	85
8	990	1110	40	4.16	4.35	3.27	16.8	16.0	0.96	0.89	29	57
9	99	1110	40	5.19	4.61	3.63	16.8	17.2	0.81	0.83	95	62
10	990	111	40	4.12	3.86	2.73	16.9	16.4	0.84	0.79	34	22
1-10				5.23	4.71	3.45	16.9	16.2	0.76	0.80	81	64

[a] Ca was applied at the rate of 1000 lb. per acre in all treatments; 570 lb. was from $CaCl_2$ in treatment 1 and 1000 lb. in 3, 6, 10; the balance was from $CaSO_4$.
[b] Cl from $CaCl_2$ was 500 lb. per acre in treatment 1 and 891 lb. each in treatments 3, 6, 10; all other Cl was from KCl.
[c] K from K_2SO_4 was 560 lb. in treatment 1 and 1000 lb. each in treatments 2, 5, 9; all other K was from KCl.
[d] N was from $(NH_4)_2SO_4$ in all treatments.

was possible through the intact tissues of the leaves. The plants in this test were placed in an inverse position in 4-gal. porcelain crocks containing 1 per cent sodium chloride. The distal third of the leaves were submerged in the salt solution for 18 hours. Analyses showed the submerged leaf tissues contained 0.9 to 2.4 mg Cl per g of fresh weight and that the nonsubmerged tissues contained 0.5 to 1.0 mg.

Control measures.—It has been adequately demonstrated that windbreaks of any kind offer partial control of leaf-tip necrosis. Plantings of *Casuarina equisetifolia* at short distances in multiple rows on the seaward side of pineapple fields have reduced leaf-tip necrosis appreciably (20). *C. equisetifolia* is able to withstand, without injury, great amounts of salt, which often appear as incrustations on the leaves. The damaging effects of chlorides may be overcome partially by ample applications of nitrogen and potassium, which stimulate vigorous plant growth and increase fruit yields appreciably.

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INHIBITION BY HIGH TEMPERATURE OF INFECTION BY TOBACCO MOSAIC VIRUS [1]

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SUMMARY

Tobacco mosaic virus did not become established in tobacco leaves that were inoculated at 38–40°C and then incubated at the same temperature for 6 hours or longer. No increase of virus occurred in such leaves during subsequent incubation at 15°. Comparable leaves inoculated at lower temperatures and then incubated at 38–40° became infected. Virus increase was inhibited in infected leaves during the heat treatment, but was resumed during subsequent incubation at 15°.

Many studies have been made concerning the effect of high temperatures on the production of viruses in infected plant tissue (2, 3, 4). Little is known, however, regarding the effect of high temperatures on the infection process. It has been demonstrated that tobacco mosaic virus accumulation in tobacco leaves is adversely affected at temperatures above about 38°C (6). This paper reports the results of work which indicate that similar temperatures prevent the establishment of the virus infection.

EXPERIMENTAL PROCEDURE.—Excised leaves, 4 to 6 in. in length, of tobacco (*Nicotiana tabacum* L. var. Havana 38) were inoculated while submerged in tobacco mosaic virus inoculum held at 38–40°C. The leaves were held in this inoculum for at least 10 minutes prior to rubbing in order to assure the proper leaf temperature.

After inoculation, and while still submerged in the inoculum, the leaves were transferred to preheated glass jars. The jars were inverted while still submerged, and the inoculum was removed by displacement with warm air. The jars were then sealed while still inverted in the inoculum and were then transferred quickly to a large water bath at the same temperature. They were kept submerged in the water bath for the duration of the heat treatment. A similar set of leaves that served as a control was always treated in a similar manner except that the temperature was held at 24–25°.

After the heat treatment, the leaves were removed from the glass jars. The leaves, with petioles immersed in pans of water, were incubated in the light in a moist chamber at about 15°. This temperature, while obviously not the optimum for tobacco mosaic virus multiplication, was chosen in order to avoid excessive degeneration of the excised leaves during the experiment. In early experiments, individual leaves were harvested at daily intervals, and six 10-mm. discs were removed and ground in 10 ml of distilled water with a mortar and pestle. In later experiments designed to ascertain only whether infection had taken place, the leaves were sampled only after 6 days. This mixture was rubbed immediately on the leaves of plants of *Nicotiana glutinosa* L. in the greenhouse. Local-lesion counts were usually made 4–5 days later.

The assumption was made that if infection had taken place in the temperature-treated leaves, the virus would resume activity and accumulate during the later incubation period, reaching levels that could be detected readily by local-lesion counts. It first was determined that the heat treatments of the leaves did not damage permanently the "virus manufacturing" mechanisms in the cells. Experiments showed that in leaves infected at lower temperatures (e.g., room temperature or 20°), subsequent heat treatments at 40° arrested virus production; but with the resumption of favorable temperatures (15°) virus accumulation again proceeded.

EXPERIMENTAL RESULTS AND DISCUSSION. — Heat treatment for 6 hours or longer at 40°C prohibited the establishment of the virus (Table 1). Heat treatments for 3 hours or less gave erratic results and obviously did not completely prohibit infection.

In preliminary experiments in which some of the precautions outlined in the preceding section were not taken, results were erratic. Results were consistent only when such precautions against leaf temperature fluctuations were made. It appears that a temperature of about 38° is critical. When leaf temperatures dropped even momentarily below 38° within 6 hours after inoculation, some infection took place. This result was observed, for example, by merely transferring the leaves through the air from the inoculum to the high-temperature water bath.

TABLE 1.—*Infection by tobacco mosaic virus of tobacco leaves that were held for various periods of time at 40° or 25°C during and after inoculation, then incubated at 15°C for 6 days*

Number of experiments	Length of temperature treatment in hours	Average number[a] of leaves infected following treatments at	
		40°C	25°C
2	24	0/7	7.5/10
7	6	0/5	3/5
3	3	1.7/5	3/5
5	1	3.2/5	3.2/5
2	0.5	4/5	3.5/5

[a] Numerator is number of leaves infected, and denominator is number of leaves inoculated.

[1] Accepted for publication July 11, 1955.
Published with the approval of the Director as Paper No. 698, Journal Series, Nebraska Agricultural Experiment Station. This paper is based upon work sponsored in part by the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland.

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Previous work (6), confirmed by preliminary experiments in this study, has indicated that a temperature of around 38° stops the accumulation of tobacco mosaic virus in infected tissue. Results reported here indicate that the same temperature prohibits the completion of the infection process. This suggests the possibility that the 2 processes may be partially dependent upon the same mechanisms. Since the thermal inactivation point of the virus *in vitro* (90°C or above) is much higher than the temperature treatments employed in this work, the data suggest that a more thermolabile "phase" of the virus exists during virus production and during some essential stage in the infection process.

It is unlikely that the inhibitive effect of water described in an earlier paper from this laboratory (1), can account for the observed results. The leaves were submerged in the inoculum even before they were rubbed. The water inhibition effect was observed when the leaves were treated with water before contact with the inoculum.

Concepts concerning the infection phenomena with certain bacterial viruses can be used to explain the present observations with tobacco mosaic infection. Puck et al (5) have proposed that one of the steps in the invasion of bacterial cells by viruses is the splitting of the viral desoxyribose nucleic acid (DNA) from its protein coat. This process is followed by injection of the DNA into the cell. In the case of plant viruses, the activity might also be associated with some moiety of the virus particle, such as the nucleic acid, which might be separated from the protein fraction. In this state the nucleic acid may be quite thermolabile. It is realized, however, that the heat treatments reported herein may influence the virus activity by affecting directly the host. It is believed, nevertheless, that this evidence supports the hypothesis that the tobacco mosaic infection process proceeds along lines similar to those now recognized for bacteriophages.

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SPECIFICITY OF BACTERIOPHAGES OF XANTHOMONAS PRUNI [1]

A. Eisenstark and L. B. Bernstein

SUMMARY

Two hundred and twelve strains of *Xanthomonas* were surveyed for susceptibility to a bacteriophage of *X. pruni*. The only ones lysed were 13 strains of *X. pruni*. Three strains of *X. pruni* failed to show lysis, but they were found upon further testing to be naturally lysogenic organisms. It is therefore concluded that the bacteriophage used is specific. A mixture of 6 phage types was used in the testing procedure. When the 13 susceptible strains were tested with each individual phage type, the strains varied in the range of their lysis.

Thornberry et al (8) pointed out that the phage-lysis technique might be used to identify plant pathogenic bacteria, particularly *Xanthomonas pruni* (E. F. Sm.) Dows. The phage preparation used resulted in specific lysis of *X. pruni* but gave negative or inconclusive results with other species. Their method consisted of turbidity comparisons of control broth cultures of bacteria with cultures to which phage had been added. Lysis was indicated by a reduction in turbidity.

Since that time, a routine procedure has been developed whereby lysis of *X. pruni* may be detected conveniently in agar petri plates (2, 4). In comparison with a turbidity test, this technique has several advantages: it gives clear-cut positive or negative re-

[1] Accepted for publication June 27, 1955.
Contribution No. 305, Department of Bacteriology, Kansas Agricultural Experiment Station, Manhattan. The authors wish to express their sincere thanks to M. P. Starr and associates, University of California at Davis, for supplying Xanthomonas cultures from the UNESCO collection; without their assistance such a broad screening of cultures would have been difficult.
These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Kansas State College, NR 135-172.

actions (Fig. 1), it avoids false reactions due to bacteriocidins or to nonspecific turbidity alterations, it is faster, and it is standard for phage typing of animal and human pathogens.

This study used such an agar-plate method to test the specificity of phages of *X. pruni* against 212 Xanthomonas cultures.

MATERIALS AND METHODS.—Unless otherwise designated, the Xanthomonas cultures were from the UNESCO collection, Department of Bacteriology, University of California, Davis. Before the screening of organisms was initiated, cultures that obviously were contaminated or that did not give the typical colony appearance of Xanthomonas were eliminated. Cultures were designated by genus and species names given to them by contributors.

The cultures screened for phage susceptibility were *X. alfalfae* (Riker et al) Dows., *X. amaranthicoli* Patel et al, *X. badrii* Patel et al, *X. barbareae* Burk. (8 strains), *X. beticola* (E. F. Sm., Brown & Town.) Savul. (3 strains), *X. cajanii* Kulkarni et al, *X. Campestris* (Pam.) Dows. (13 strains, 1 supplied by J. O. Harris, Kansas State College), *X. carotae* (Kendr.) Dows. (3 strains), *X. cassavae* Wiehe & Dows., *X. cassiae* Kulkarni et al, *X. clerodenroni* Patel et al, *X. corylina* P. W. Miller et al (5 strains), *X. cucurbitae* (Bryan) Dows., *X. desmodii* Uppal & Patel, *X. desmodii-gangeticii* Uppal et al, *X. dieffenbachiae* (McColloch & Pirone) Dows. (4 strains), *X. erythrinae* Patel et al, *X. geranii* (Burk.) Dows. (6 strains), *X. gummisudans* (McCull.) Starr & Burk. (3 strains), *X. hederae* (Arnaud) Dows. (3 strains), *X. holcicola* (Elliot) Starr & Burk. (3 strains), *X. hyacinthi* (Wakk.) Dows. (4 strains), *X. incanae* (Kendr. & Baker) Starr & Weiss (9 strains), *X. juglandis* (Pierce) Dows. (23 strains), *X. lactucae* (Yamamota) Dows. (2 strains), *X. lactucae-scariolae* (Thornberry & Anderson) Savul. (2 strains), *X. lawsoniae* Patel et al, *X. lespedezae* (Ayers et al) Starr (2 strains), *X. macurlafolium-gardeniae* (Ark.) Elrod & Braun (2 strains), *X. malvacearum* (E. F. Sm.) Dows. (12 strains, 1 supplied by L. A. Brinkerhoff, Oklahoma A. and M. College, Stillwater.), *X. manihotis* (Arth.-Ber.) Starr (13 strains), *X. melhusi* Patel et al, *X. nigromaculans* f. sp. *zinniae* Hopkins & Dows., *X. papavericoli* (Bryan & McWhorter) Dows. (4 strains), *X. pelargonii* (Brown) Starr & Burk. (8 strains), *X. phaseoli* (E. F. Sm.) Dows. (20 strains), *X. poinsettiaecola* Patel et al, *X. pruni* (E. F. Sm.) Dows. (16 strains, 2 from The American Type Culture Collection, No. 10016 and 10017, and 5 isolated by H. H. Thornberry, University of Illinois, Urbana.), *X. ricinicola* (Elliot) Dows. (2 strains), *X. rubrilineans* (Lee et al) Starr & Burk. (3 strains), *X. stizolobiicola* Patel et al, *X. tamarindi* Patel et al, *X. translucens* f. sp. *cerealis* Hagborg (16 strains), *X. uppalii* Patel, *X. vasculorum* (Cobb) Dows. (8 strains), *X. vesicatoria* (Doidge) Dows. (17 strains), *X. vignicola* Burk. (2 strains), *X. vitians* (Brown) Starr & Weiss (2 strains), 2 unidentified Xanthomonads pathogenic to plantain (*Plantago lanceolata* L.), and 4 pathogenic to dandelion (*Taraxacum officinale* Weber).

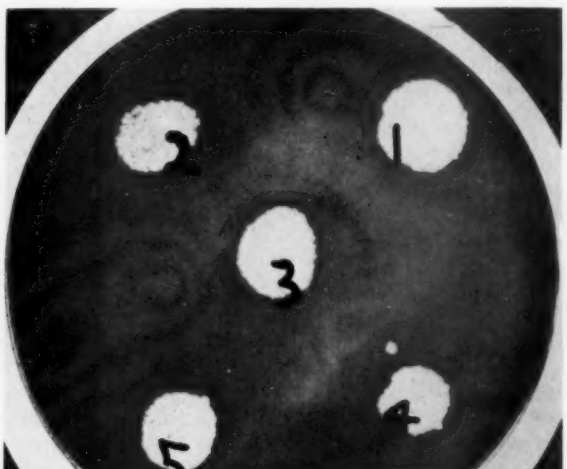

FIG. 1. Lysis of *Xanthomonas pruni* by specific bacteriophage. A positive test is indicated by a clear zone at the spot where phage was dropped on the surface of a plate that had been seeded with bacteria. The numbers on the petri plate indicate the phage types used to produce the lysis.

Nutrient-broth cultures were prepared for each strain used in the screening program. From each of these broth cultures, 3 ml containing approximately 10^8 actively growing bacterial cells was incorporated into a thin top layer of a nutrient-agar plate (2). This was done by first preparing a regular nutrient-agar plate and then adding a second layer consisting of a mixture of 3 ml of bacterial cells and 2 ml of nutrient agar.

The phage preparation used consisted of a mixture of each of the pure stocks that previously have been isolated and designated as Xp1, Xp2, Xp3, Xp4, Xp5, and Xp8 (3). The mixture was used to avoid the possibility that an organism might be resistant to one of the phage types but not necessarily to all (3). One drop of the mixture was placed on the surface of each plate. If an organism was lysed by the mixture, it was further tested with each of the Xp phages individually. After 24 hours of incubation, plates were examined for clear spots at the sites of phage addition. A seeded petri plate to which droplets of 5 of the phage types had been added is shown in Figure 1.

In cases where cultures of *X. pruni* failed to show lysis upon addition of phage, strains were tested for lysogenicity, since a lysogenic strain of *X. pruni* isolated from previous studies (1) was known to be resistant to these phage types. A drop of filtrate from a broth culture of the suspected strain of *X. pruni* was added to an agar plate seeded with a strain of *X. pruni* known to be susceptible to the lysogenic phage strain. If the suspected strain of bacteria had harbored phage, a clear spot should have appeared, after incubation, at the site where the filtrate was added.

RESULTS.—With the exception of the cultures of *X. pruni*, no lysis was obtained with any of the strains tested. Thirteen of the 16 cultures of *X. pruni* were lysed by the phage; the remaining 3 showed no signs of lysis. The nature of the clear zones may be seen in Figure 1. The 3 strains that failed to show lysis were found upon further testing to be naturally lysogenic organisms.

The 13 strains of *X. pruni* lysed by the mixture of phages were further tested individually with each of the Xp phages (Table 1). In some cases, lysis was indicated by zones that were not completely clear. Such partial lysis has been described previously (3). As shown in Table 1, some cultures isolated from natural infections show a range of response to bacteriophage types resembling those that may be obtained in the laboratory by a selection process (3).

TABLE 1.—*Bacteriophage lysis range of isolates of X. pruni*[a]

Number and source of isolate	Lysis by phage type[b]					
	Xp1	Xp2	Xp3	Xp4	Xp5	Xp8
143, UNESCO	−	−	+	VW	−	+
110, UNESCO	+	+	+	+	+	+
13, UNESCO	−	−	+	W	VW	+
109, UNESCO	−	−	+	W	−	+
11, UNESCO	+	+	+	+	+	+
103, UNESCO	+	+	+	+	+	+
10016, ATCC	+	+	+	+	+	+
10017, ATCC	+	+	+	+	+	+
1, Thornberry	+	+	+	+	+	+
2, Thornberry	+	+	+	+	+	+
3, Thornberry	+	+	+	+	+	+
4, Thornberry	+	+	+	+	+	+
5, Thornberry	+	+	+	+	+	+

[a] UNESCO isolates 114, 115, and 101 were negative throughout.

[b] + Indicates a positive reaction characterized by a completely clear zone as illustrated in Figure 1; − indicates no lysis; W indicates that cleared zones with some overgrowth were formed; and VW that the lysis was very faint.

DISCUSSION.—Use of bacteriophages to type bacterial organisms has been particularly useful where closely related organisms are difficult to distinguish by other methods. Organisms that fall into this category are those of the genera *Salmonella*, *Staphylococcus*, and *Rhizobium*. Species of *Xanthomonas* are difficult to distinguish by the usual determinative tests, and some of them, such as *X. pruni* and *X. lactucae-scariolae*, are reported to be distinguishable only by pathogenicity tests (6). For this reason, the development of a specific phage-typing procedure seemed desirable. In the particular case of the 2 organisms mentioned above, Thornberry et al (8) have pointed out that a phage of *X. pruni* lysed only its specific host; this is verified in the present study where the 2 strains of the related organism *X. lactucae-scariolae* failed to lyse.

Inasmuch as isolates from diseased plants may not be lysed by each of the phage types, a mixture of phages would be advantageous for typing procedure. If it were desirable to type *X. pruni* into substrains, the use of specific phage types, rather than the mixture, might be a useful approach.

Katznelson and associates (5, 6) have used phage-typing procedures for other species of *Xanthomonas* with varying degrees of success.

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FUSARIUM WILT OF CATTLEYAS[1]

Virginia Foster

SUMMARY

A fungus on Osmunda root fibers was found to be the cause of a wilt disease of Cattleya orchids. This fungus is classified in the genus *Fusarium* in the Elegans section of Wollenweber and Reinking. It penetrates past the digestion cells into the stele of the orchid roots and colonizes the roots, rhizomes, and pseudobulbs. Symptoms of the disease occur 3–9 weeks following inoculation. Flowering is especially affected. There are fewer, smaller, and shorter-lived flowers on inoculated plants than on noninoculated ones.

Different temperatures, humidities, and day-lengths during a 48-hour incubation period had no apparent effect on disease development in inoculated Cattleya plants.

Culture filtrates of 14 single-spore isolates were studied for biological activity. The culture solutions increased in alkalinity up to the 100-150th day of growth of the fungus, and then declined slightly in alkalinity. Culture filtrates collected after certain periods of fungus growth caused increased oxygen absorption of orchid leaf tissue; filtrates collected at other intervals caused decreased oxygen absorption. Variability among the isolates was shown by the differences in age of culture at which changes in oxygen absorption occurred.

An alcohol-soluble, thermostable portion of the culture filtrates caused wilting of Bonny Best tomato plants. The wilting toxin appeared in culture filtrates of all the 14 fungus isolates, but the time of appearance varied with the isolate. An alcohol-insoluble, thermostable portion of the culture filtrates caused curling of tomato leaflet edges and browning of the leaflet blades.

Two orchid plants of the genus *Cattleya* Lindl. in a private collection wilted, then the leaves abscised, and the roots decayed. Later, the pseudobulbs and rhizomes died. The plants had been healthy until changed from potting material of sphagnum moss to Osmunda fibers.

Microscopic examination revealed the presence of septate hyphae in and among the cells of the roots, the rhizomes, and the pseudobulbs. Fungus infection of a mycorrhizal type is ordinarily present in orchid roots, but tubers, stems, and leaves normally are not infected.

ISOLATION AND IDENTIFICATION OF THE PATHOGENIC FUNGUS.—Pieces of orchid tissue and the Osmunda fibers were surface-sterilized by 3 methods: 1) soaking 10 minutes in 1:1000 $HgCl_2$, then rinsing 3 times in sterile water; 2) soaking 3 minutes in 3 per cent Chlorox (5 per cent sodium hypochlorite) then rinsing in sterile water; and 3) flaming sections dipped in ethyl alcohol. The surface-sterilized material was then plated out on 6 types of sterile agar in petri plates, 5 pieces to a plate.

A Fusarium with tufted pink aerial mycelium appeared on all types of agar and from all kinds of tissue used, but not from all fragments. Of the 200 fragments used, approximately 100 proved to have this fungus in them; 15 fragments had a white fungus with clamp connections, 6 had a yellow Aspergillus with black spore heads, 3 had a green Penicillium, and 11 fragments had bacteria. No microorganisms grew from the other fragments.

Fourteen single spores of the pink Fusarium, obtained with a De Fonbrune micromanipulator, were viable. These and a mass transfer culture were cultivated on Difco yeast-extract agar, potato-dextrose agar, steamed potato, and steamed rice. The potato and rice cultures were exposed to daylight throughout the growth period, which was in July and August. The macroconidia on steamed potato were longer than those in species closely resembling this fungus in other ways. Harter (4) had found that Fusarium conidia were considerably longer in cultures grown in the light than in the dark. The macroconidia ranged from 4.5 to 8 μ in width and from 47 to 98 μ in length. Macroconidia were found in not more than 3 per cent of the total number of spores. There were more macroconidia in the cultures from isolated macroconidia than in cultures from isolated microconidia.

After 10 days' growth, the cultures on steamed rice were shell pink, as judged by Ridgway's standards (8). After 4 weeks' growth, there were areas varying from pale vinaceous pink, light vinaceous lilac, or deep lavender to dull blue violet and dark vinaceous purple.

On 3-month-old potato-dextrose-agar plates, cultures of all the isolates and the mass culture had terminal and intercalary chlamydospores; verticillately branched conidiophores, sometimes in sporodochia; and anastamosed mycelial strands bearing conidia on side branches.

This fungus closely resembles the description of *Fusarium orthoceras* Appel et Wr., var. *longuis* (Sherb.) Wr. (12). A classification of it according to the system of Snyder and Hansen (9) would place it as being *Fusarium oxysporum* Schlecht, f. *cattleyae*.

PATHOGENICITY.—Bits of mycelium of the 4 types of

[1] Accepted for publication June 30, 1955.

Paper No. 580, Department of Botany and Plant Pathology, The Ohio State University, Columbus, Ohio. From a dissertation submitted in partial fulfillment of the requirements for a Ph.D. degree in the Graduate School of The Ohio State University.

fungi found on the plates implanted with diseased tissue fragments were inserted in wounds made with sterile knives in the pseudobulbs of 8 Cattleya plants of blooming age. Sterile cotton was bound over the wounds and the plants kept for 48 hours in a moist chamber at 100 per cent relative humidity and at 75°F. There were no immediate results apparent from these inoculations. Two years later, the 2 plants that had been inoculated with the Fusarium died of leaf wilt and root rot. The other plants remained healthy.

Other inoculations were made by pouring a spore suspension of the Fusarium in 1 per cent orange juice over the roots of 3 Cattleyas of blooming age and by binding nonsterilized Osmunda fibers from the stock used as potting material for the 2 original plants about the roots of 3 other Cattleyas of blooming age. Four check plants were treated with sterile distilled water. Leaf wilt appeared 3–9 weeks after inoculation. All of the inoculated plants died of leaf wilt and root rot. A Fusarium that appeared identical to the original was recovered from the diseased tissues (Fig. 1).

Cultures of 3 of the single-spore isolates were used in root inoculations of 9 Cattleya plants potted in quartz gravel mixed with a small quantity of Osmunda fibers. Three inoculated plants and 1 check plant were then placed in each of 3 different environments for 48 hours. These environments were 1) a moist chamber in which temperature was maintained at 75°F and relative humidity at 100 per cent, 2) a room in which 12-hour periods of darkness (60°, 60 per cent relative humidity) were alternated with 12-hour periods of light (75°, 90 per cent relative humidity), and 3) a greenhouse bench on which the plants were kept before inoculation and to which all the plants were returned after the 48-hour incubation period. During the incubation period on this bench, the relative humidity was 34–95 per cent, and the temperature 60–97°.

None of these plants have died in 20 months. Leaves of the inoculated plants became yellow and wrinkled, but those that did not abscise regained their original glossy green appearance. The inoculated plants had more dead flower buds; fewer, smaller, and shorter-lived flowers; and fewer new leaves than did the check plants. The different environments during the incubation period of 48 hours had no apparent effect on disease development. The isolates used in this test were less pathogenic than those used in earlier inoculations.

Sections of Cattleya roots free of internal microorganisms were surface-sterilized and placed on mycelial mats of the Fusarium isolates in petri plates. Control sections were placed on sterile agar. At intervals, sections were removed from the plates, fixed in a mixture of formalin and propionic acid, imbedded in paraffin, cut in 10-μ sections, and stained with safranin and picric acid plus aniline blue.

The Fusarium hyphae penetrated into the cortical parenchyma of the root pieces in 1 day; in 3 days, hyphae had entered the stele through passage cells in the endodermis. No hyphae were found in the sections placed on sterile agar.

FIG. 1. Cattleya plants photographed 1 year after inoculation. Plant on left is noninoculated check; plant on right was inoculated with Fusarium from Osmunda fibers used in potting the original 2 plants.

PHYSIOLOGICAL STUDIES OF FUNGUS CULTURE FILTRATES.—There are many reports in the literature pertaining to physiological activity found in culture filtrates of plant pathogenic Fusaria (3, 5, 7, 10, 11). Since the orchid wilt Fusarium was taxonomically so close to other Fusaria that produce toxic substances in culture, it seemed desirable to investigate the possible biological activity of culture filtrates of this organism.

Preparation of culture filtrates.—A small amount of mycelium from the center of 5-day-old slants of the 14 isolates and of the mass culture were transferred to 200 ml of modified Richard's solution (modification of Luz (5)) in 500-ml Erlenmeyer flasks. The pH after sterilization for 20 minutes at 15 lb. pressure was 4.0. The cultures were kept in a dark room at 75°F ± 3°.

At intervals filtrates were removed from the flasks and filtered through Whatman no. 30 filter paper by gravity filtration. This method successfully removed all spores and bits of mycelium.

After 10 days, the pH of cultures of the 14 isolates and the mass culture ranged from 5.0 to 5.6; at 20 days, from 5.8 to 6.6; at 30 days, from 6.1 to 6.9; at 50 days, from 6.7 to 7.5; at 70 days, from 7.0 to 7.7; at 90 days, from 7.0 to 7.8; at 140 days, from 7.3 to 7.9; at 170 days, from 7.1 to 7.6; at 250 days, from 6.5 to 7.3; and at 300 days, from 6.4 to 6.9.

Effect of filtrates on oxygen absorption by orchid leaf tissue.—One ml of green healthy Cattleya leaf tissue, sliced into cubes approximately 1 mm square, was placed in 1 ml of a 0.02 M phosphate buffer (pH 7) in the reaction space of Warburg flasks with center wells and side arms. In the center wells was placed

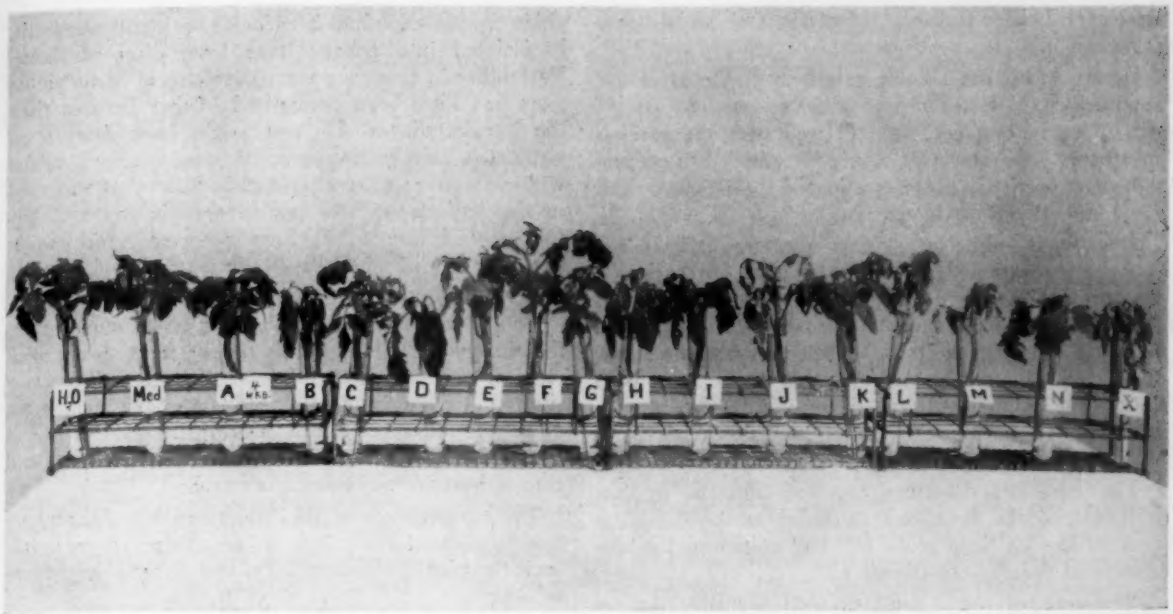

FIG. 2. Excised shoots of Bonny Best tomato plants after 24 hours with cut stems immersed in (left to right) distilled water, sterile media, 4-week filtrates from cultures A–N, and 4-week filtrate from a mass culture.

20 per cent KOH solution to absorb carbon dioxide. One ml of a fungus culture filtrate or of the sterile culture media was placed in the side arm of each vessel.

The filled flasks were immersed in the 28°C water bath of a Warburg constant-volume respirometer for 30 minutes, then the manometers were closed and readings taken of the oxygen absorption of the leaf tissue in the buffer solution. The filtrates were then dumped from the side arms and readings taken for an hour at 5-minute intervals. Several tests were made with filtrates at different ages.

A sharp decline in oxygen absorption by the leaf tissue occurred when the culture filtrates were first added. Ten to 15 minutes later, the absorption in the filtrates did not differ significantly from the absorption in the sterile culture media.

In triplicate tests made of culture filtrates 10 days old, the total oxygen absorption of leaf tissue in filtrates of isolates A, B, C, D, E, I, J, K, L, M, N, and the mass culture was less than in the sterile culture solution; the total oxygen absorption in filtrates of isolates F, G, and H was greater. At 20 days, oxygen absorption in filtrates of isolates A, B, C, D, and E was less; in filtrates of isolates F, G, H, I, J, K, L, M, N, and the mass culture oxygen absorption was greater. At 30 days, oxygen absorption in filtrates of isolates A, B, C, D, E, F, K, N, and the mass culture was less; in filtrates of isolates G, H, I, J, L, and M oxygen absorption was greater.

Effect of filtrates on tomato plants.—Tomato seedlings (var. Bonny Best) 10–12 in. tall and with about 4 leaves, were cut off at the roots under water, allowed to become equilibrated for an hour in distilled water in the controlled temperature and light room in which the experiments were made, then placed in test tubes holding 15–20 ml of the culture filtrates. Two checks were run with each experiment: a tube of distilled water and 1 of sterile media. At the end of 24 hours, the plants were removed from the filtrates, washed with tap water and then distilled water, placed in tubes of distilled water, and returned to the experimental room for an additional 24 hours.

The tomato plants were affected 3 ways: 1) curled-up edges of leaflets (J, K, L, and X in Fig. 2); 2) dark spots on leaflet blades (J, K, L, and X in Fig. 2); 3) wilt of stem and petioles (B, D, I, J, and X in Fig. 2). Filtrates of all of the isolates at some culture age caused all 3 effects in the tomato plants, but the isolates differed with respect to the age of the culture that produced the toxic effects. None of the wilted plants recovered.

Solution culture filtrates of 3 of the Fusarium isolates were extracted with aldehyde-free 95 per cent ethyl alcohol. A cloudy white precipitate formed. Both the alcoholic supernatant and a water solution of the precipitate were vacuum distilled at 35°C. The residues were made up to the original volume of filtrate used and placed in test tubes in 15-ml portions. For controls, 15-ml portions of unextracted filtrates of the three isolate cultures, of sterile media, and of distilled water were prepared. One tube of each set was heated to 100°C in a water bath for 15 minutes. Bonny Best excised shoots were then placed in all the solutions for 24 hours.

No toxic effects appeared in the sterile media solutions or the water. In both heated and unheated unextracted filtrates, curled up leaflet edges, dark spots

on leaflet blades, and wilt of stems and petioles occurred.

In tests with the residue solutions of the alcoholic supernatant, both heated and unheated portions caused wilt of stems and petioles. In tests with the residue solutions of the alcoholic precipitate, both heated and unheated portions caused curling of leaflet edges and dark spots on leaflet blades.

DISCUSSION.—The form genus *Fusarium* is the longest-cited form genus in connection with mycorrhizae. In most of the early attempts to isolate the fungi from orchid mycorrhizae, Fusaria were obtained. In 1904, Bernard (1) decided that Fusaria are not the fungi that stimulate germination of orchid seed. His observation has been substantiated by other workers. Fusaria have been found living on organic debris from the soil all over the world.

The Fusarium described herein is unusual in that it withstood the fungitoxic material in the digestion cells of the Cattleya roots (2) and colonized the rhizome and pseudobulb tissue.

Virulence of the 3 isolates used for the last described inoculations of Cattleya plants was less than that of the cultures used in earlier inoculations. These 3 cultures had undergone several successive transfers on artificial media before being used as inoculum. Other inoculations had been made with cultures freshly isolated into pure culture from diseased tissue. Variability in virulence among isolates of plant pathogens has long been recognized. Other isolates than the 3 selected for this test might have been more virulent. The potting mix of quartz gravel mixed with a small quantity of Osmunda fibers that was used in this experiment was low in organic content. McClellan and Stuart (6) have reported a decline in pathogenicity of other Fusaria in tests of environments with low organic content.

Data obtained with the Warburg respirometer show that in culture solutions this fungus forms some principle or principles that depress respiration. They also show that other metabolic products of the fungus act as respiration accelerators. During the growth period of the fungus there are changes in kind, amount, and rate of formation of these compounds.

The toxic effects of the fungus culture filtrates on tomato plants further indicate that there are materials toxic to plants elaborated by this fungus in culture solutions.

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ELECTRON MICROSCOPY OF TOBACCO MOSAIC VIRUS IN SITU[1]

C. B. Skotland, D. J. Hagedorn, and Mark A. Stahmann

SUMMARY

Ultrathin sections of healthy plant tissue and of tissue infected with tobacco mosaic virus were examined in the electron microscope. Cell sections from infected tissues showed evidence of frequent chloroplast disintegration and the release of the grana and other chloroplast contents into the cytoplasm. Virus, visible as rod-shaped particles, was observed most frequently in regions containing these grana. This suggested an association of these chloroplast components and virus formation. No difference between the nuclei of diseased and healthy tissue was found. Bodies which have been interpreted as X-bodies were found.

Considerable evidence has been presented in recent years that indicates an association of virus particles with chloroplasts of plant cells. Black et al (1), in visualization of tobacco mosaic virus particles within infected cells, frequently observed such particles associated with chloroplasts. Kausche and Ruska (6) and Leyon (8) have also presented evidence that emphasizes a close relationship between virus particles and chloroplasts. Smith (14), however, did not find any such association of tomato bushy stunt virus or turnip yellows virus particles with chloroplasts in ultrathin sections of plant tissue.

MATERIALS AND METHODS.—Ultrathin sections (0.5–1.5 μ in thickness) of both diseased and healthy tobacco leaf tissue (*Nicotiana tabacum* L. var. Havana 38) were studied in an R.C.A. model EMU2A electron microscope equipped with an object aperture. The tissue sectioned was taken from plants that had been infected with a typical strain of the tobacco mosaic virus supplied by Dr. R. W. Fulton. There were no apparent differences in the number or location of virus particles from plant tissue infected for from 2 to 8 weeks.

The tissue was fixed in FAA and passed through a closely graded ethanol dehydration series. The final embedding in n-butyl methacrylate was carried out according to the procedure described by Newman et al (11). Sections were cut with a Spencer rotary microtome fitted with the thermal expansion apparatus described by Newman and co-workers (11). A glass edge (7) was used in lieu of the conventional microtome blade.

The sections were removed by means of a camel's-hair brush and were placed on glass microscope slides in drops of a 25 per cent solution of dioxane in water (5). The sections flattened on this solution. After the dioxane and water had evaporated, the glass slide was washed with amyl acetate to remove the methacrylate. The slide was then flooded with a 2 per cent solution of parlodion in amyl acetate and the excess drained off. This formed the membrane in which the plant tissue was embedded. The parlodion membrane and plant tissue were floated off the glass slide onto water. The electron microscope screen was then placed over the plant sections, and the membrane was remounted on the glass slide in such manner that the screen was between the glass slide and the membrane. The slide was desiccated over calcium chloride for 24 hours, then shadowed with uranium.

EXPERIMENTAL RESULTS. — More than 200 electron micrographs were taken. The figures discussed here were chosen as representative and are supported by photographs not included in the text and by unrecorded observations with the electron microscope. A preliminary report already has been made (13). Figure 1,A is an electron micrograph of a section of a cell from a healthy tobacco leaf. It shows a section through a single normal chloroplast. The granular nature of the chloroplast is considered to be in a large part due to the presence of grana.

Figure 1,B is a cell section from a leaf from a diseased plant. It shows several chloroplasts, some of which appear normal. Some of these can be seen as dense granular bodies in the lower right portion of this photograph. These chloroplasts contain structures of a density and shape similar to those of the granules in chloroplasts of healthy cells (Fig. 1,A). This section also shows disintegrated chloroplasts at the top of the figure. Granular material similar to that in normal chloroplasts appears to have been released from the disintegrated chloroplasts. A mass of these granules is shown embedded in an amorphous material in Figure 2,A. The amount of this material is small in areas where rod-shaped particles are numerous and large where rods are few. A portion of this amorphous material around the granules appears to have aggregated into rod-shaped particles in the cell illustrated in Figure 2,B.

Figures 2,C and 2,D show a group of dark particles surrounded by rod-shaped or fibrous particles that are considered to be tobacco mosaic virus. These rod-shaped particles are of the approximate size reported for tobacco mosaic virus. An exact determination of their size is difficult; however, the estimated width of

[1] Accepted for publication July 5, 1955.
Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School with funds supplied by the Wisconsin Alumni Research Foundation. Appreciation is expressed to Dr. Paul Kaesberg for assistance with the electron microscope and to Mr. Eugene Herrling for preparation of the figures.

the smaller particles is about 15–20 mμ. Many of the particles appear longer than the length generally ascribed to tobacco mosaic virus, but this may be due to end-to-end aggregation (1). Relatively little amorphous material is seen about these virus particles.

Figure 3 is a cross section of an infected cell showing the cell wall and several chloroplasts. The intact chloroplasts near the upper right corner are not surrounded by granules, and there are few virus particles adjacent to them. The disintegrated chloro-

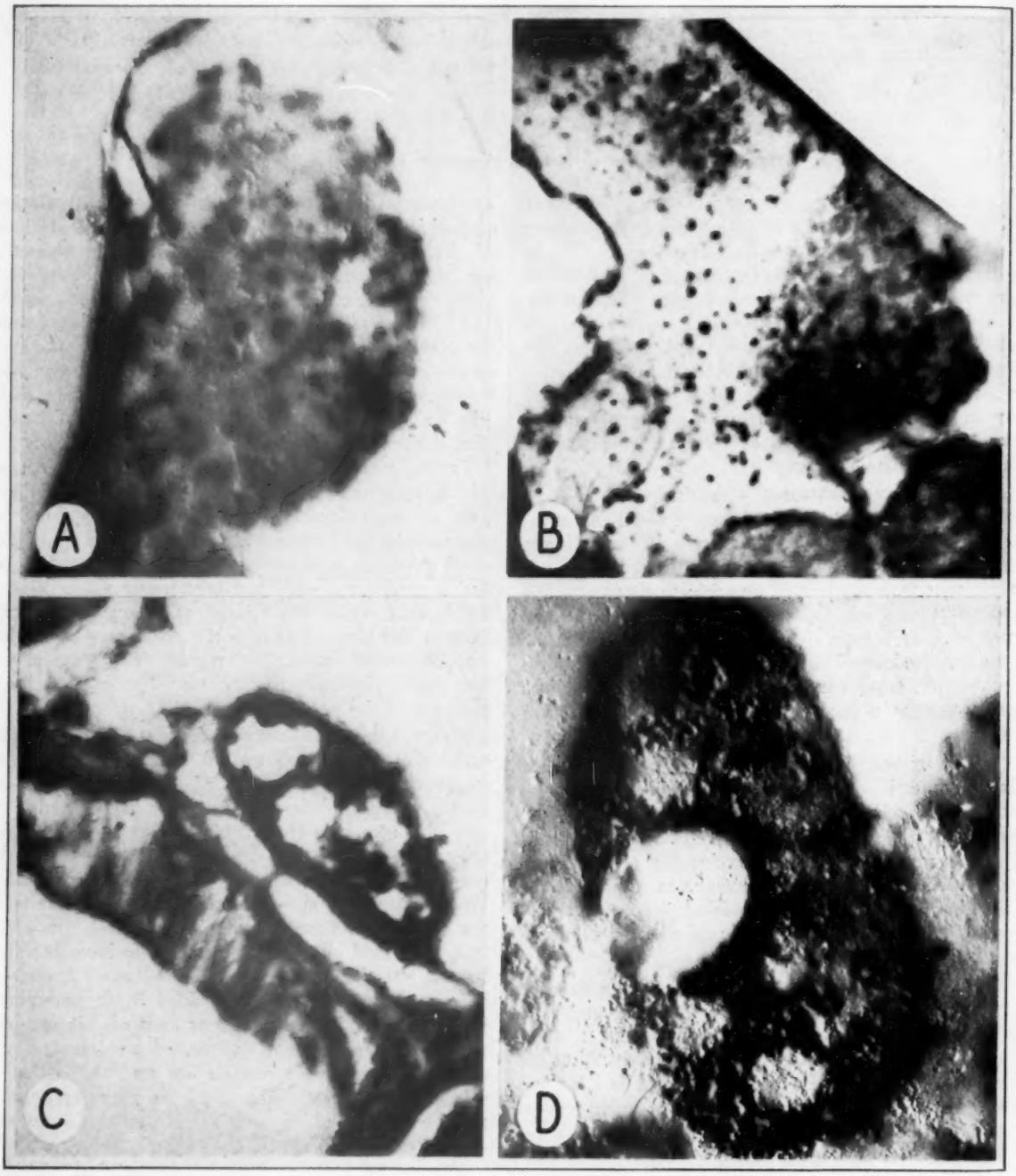

FIG. 1. A) Section through a healthy cell showing a normal chloroplast containing grana that appear as small dark particles. 4622×. B) Section through a virus-infected cell showing normal-appearing chloroplasts (lower right) and disintegrated chloroplasts (top) that have released grana that appear as small dark particles. 4471×. C) Section through a virus-infected cell showing nucleus (center right) free of virus particles and a structure of unknown function (center left). 4946×. D) Section through a virus-infected cell showing what is believed to be an X-body. 4860×. Note that neither the nucleus nor the X-body contains virus particles.

plasts on the lower and left side, however, are completely surrounded by a mass of virus particles and granules. In only 1 instance, out of more than 200, were the virus particles found adjacent to seemingly intact chloroplasts. In this case the virus particles were arranged in a parallel layer along the peripheries of adjacent sides of 2 chloroplasts.

Nuclei occasionally were observed in the sections. They appeared as dense reticulate bodies (Fig. 1, C), and the nucleolus was often present. No virus was discerned in the nuclei, and no difference was noted between the nuclei of diseased and healthy tissue. Figure 1, C also shows (center, left) a mass of material, somewhat removed from the nucleus, that is similar to the virus particles observed by Black, et al (1).

Two vacuolate bodies of a density similar to that of the nuclei were found in diseased tissues. These bodies (Fig. 1, D) appeared as dark granular structures that resembled the X-bodies as seen through the light

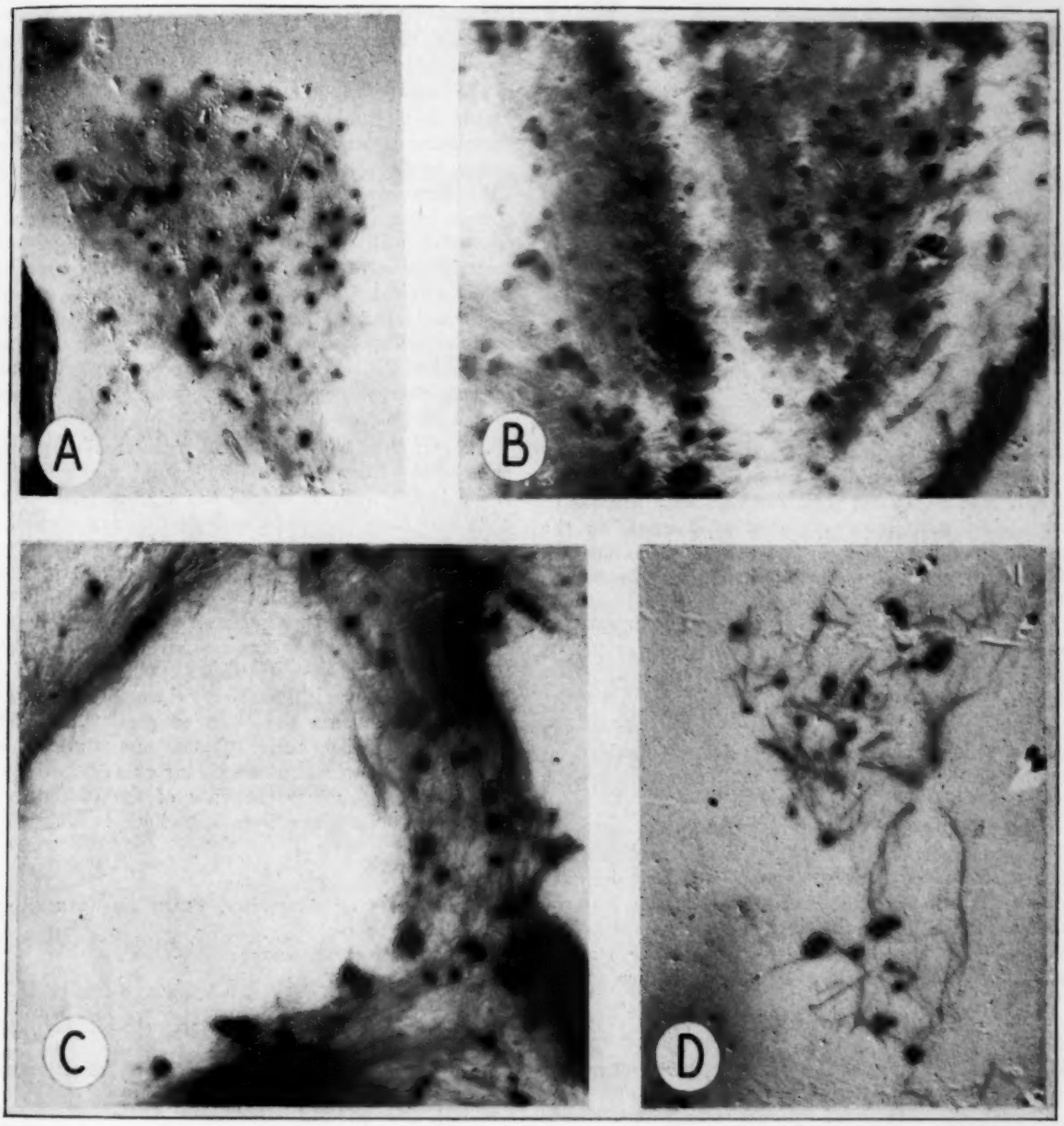

FIG. 2. A) Section through a virus-infected cell showing a mass of grana embedded in amorphous material. 4233×. B) Section through an infected cell showing grana embedded in amorphous material that seems to be partially aggregated into rod-shaped particles suggestive of an intermediate stage of virus synthesis. 4860×. C and D) Sections through virus-infected cells showing grana surrounded by rod-shaped particles believed to be completed virus particles. 7922× and 5400×. Note relative absence of amorphous material.

FIG. 3. Section through a virus-infected cell showing the cell wall on the outside, normal-appearing chloroplasts at upper right, and disintegrated chloroplasts surrounded by free grana and virus particles at lower left and center. 2195×.

microscope in prepared slides of infected tobacco tissue that were furnished by Dr. R. W. Fulton. It could not be determined from our electron micrographs whether or not virus particles were situated in the X-bodies, but virus particles appeared in the proximity of 1 of these bodies (Fig. 1, D). Sheffield (12) reported the presence of virus particles within X-bodies in her studies with the electron microscope.

DISCUSSION.—A characteristic feature of sections of cells from diseased tissues was the presence of disintegrated chloroplasts and many small dark particles dispersed throughout large portions of the cell. Sections from healthy cells contained such particles only within intact chloroplasts. This feature enabled us readily to distinguish virus-infected cells from healthy cells.

The grana found in healthy chloroplasts were not sharply defined. Their indistinct margin probably was due to the solvent action of ethanol during dehydration on the lipoids and other ethanol-soluble material found in the grana as reported by Granick and Porter (4). These workers found that the margins of spinach chloroplasts became indistinct when washed with methanol. Grana have been demonstrated to be disc shaped and of laminated construction (9, 15, 17).

The dark granules found dispersed in many cells probably had been released from chloroplasts as a result of the action of the virus. Since these granules were never found outside chloroplasts in healthy tobacco tissue, it was considered that the virus infection caused this disintegration. These granules resembled the grana found in chloroplasts from healthy tissue in size, shape, and density. On this basis the authors have concluded that these granules were remains of grana, or the stromatic spherical inclusions of high electron density observed by Cohen and Bowler (2) in their study of tobacco chloroplasts.

The nature of the amorphous material seen in Figure 2, A is unknown. It is possible that it was the proteinaceous stroma of the chloroplasts (16). Recently, by use of the electron microscope, Thomas et al (15) demonstrated the presence of grana in such a stroma from spinach choloroplasts. Our results are consistent with this. Whether or not the amorphous material is used to form a part of the virus particles is not known. One possible interpretation of Figure 2, B would suggest that the amorphous material was an intermediate stage of virus synthesis, for in some cases (Fig. 2, C, D) the grana are surrounded by the fibrous masses commonly associated with tobacco mosaic virus.

Support for such a possible explanation of the association of the virus particles and grana can be found in the work of Woods and DuBuy (18). They state "the virus may be formed from the same building units for chromoproteins (or its hydrolytic products) if not from the chromoprotein itself." On this basis and from our observations it seems possible that the proteins found in or associated with the grana are transformed by some means into virus particles. Both the grana (14) and mitochondria (10) are known to contain proteins. It has been widely accepted that the various plastids and included grana have originated from mitochondria. Woods and DuBuy also published cytological, genetical, and chemical evidence (3, 19) that supports the view of the evolution of phytopathogenic virus from mitochondria and their derivatives.

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SUNBURN PREDISPOSES WALNUT TREES TO BRANCH-WILT[1]

Noel F. Sommer[2]

SUMMARY

Hendersonula toruloidea Nattrass failed to infect, or at most was weakly parasitic to, normal branches of vigorous walnut trees inoculated with it. When branches even mildly sunburned were inoculated, however, extensive disease cankers developed. Severe branch-wilt damage is limited to orchards that are subjected to high summer temperatures. Here abundant infection courts may result from sunburn, and favorable temperatures for disease canker development are present. While high relative humidities were found necessary for spore germination, the required moisture evidently is provided by host tissues. The application of whitewash effectively reduced disease canker development when inoculations were made in branches exposed to the sun. Evidently the major effect of the whitewash is a reduction in the amount and severity of the sunburning which predisposes the branches to colonization by the fungus.

The branch-wilt disease of the Persian walnut tree, *Juglans regia* L., is found throughout the central valleys of California. In at least 1 important walnut-growing district, it threatens the industry. In other areas it has been locally serious and is potentially a problem wherever walnuts are grown under high-temperature conditions.

The branch-wilt disease does not commonly kill trees, but the death of individual fruiting branches decreases yields, and the cost of removing infected branches materially increases production costs. Although infection usually occurs on the smaller branches, the fungus, *Hendersonula toruloidea* Nattrass, remains alive in the cankers for several years. If the infected branches are not removed, it invades and kills larger branches. Moreover, the fungus produces numerous spores on infected branches, and these spores are readily disseminated by wind and rain.

Death of branches begins in late June and continues until September. Ordinarily the disease is not noted until the leaves wilt and die. Symptoms, however, can be detected much earlier as elongated darkened areas that extend for some distance along the upper side of a branch. These areas gradually enlarge in all directions (Fig. 1, C). Wilting and death of the branch do not occur until the canker has encircled the branch, or nearly so. Occasionally a watery exudate is present. The xylem tissues become darkened, with the discoloration extending for considerable distances beyond the limits of the canker in the bark.[3] Apparently the older nonliving xylem or

[1] Accepted for publication July 6, 1955.
Portion of a thesis submitted to the Graduate Division, University of California, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
Appreciation is expressed to Dr. E. E. Wilson, Professor of Plant Pathology, University of California at Davis, for guidance during this study.
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[3] The term "bark," as here used, refers to all tissues external to the vascular cambium.

heartwood is not affected.

A layer of spores is produced from sporodochia that develop between the phellem and the phelloderm. The phellem becomes loose and wrinkled, and gradually portions of it break away, exposing the dense black layer of arthrospores produced beneath it. This loose and wrinkled phellem, which in breaking away in certain areas exposes the black powdery spore layer, is the most reliable diagnostic feature of the disease (Fig. 1, A, B).

Research leading to the development of effective control measures has been hindered by a lack of information concerning the etiology of the diesase. This study was made to determine the mode of infection and the factors that influence disease development.

RELATION OF TEMPERATURE TO DISEASE DEVELOPMENT.—According to Wilson (4, 5), the optimum temperature for growth and spore germination of *H. toruloidea* on artificial media is 30–33°C. Growth was slow at 20° and at 38°. In tests on the temperature requirements of the fungus in walnut tissue, freshly cut branch sections, approximately 8 in. long and ¾ in. in diameter, were dipped at each end into paraffin to prevent drying. These were inoculated by cutting a hole in the bark with a cork borer 4 mm in diameter, inserting a block of potato-dextrose agar containing mycelium of the pathogen, and replacing the bark. Fifteen of these branch sections were placed on wet paper towels in each container and held at each temperature.

A preliminary test at temperatures of 12°, 18°, 24°, 27°, 30°, and 36°C resulted in very little growth except at 30° and 36°. In another test, inoculated branch sections were maintained at temperatures of 26°, 30°, 34°, 38°, 42°, 46°, and 50° for 10 days. Since the branch tissue dies at 42° or above and progress of the fungus could not be determined visually, isolations were made at different distances above the point of inoculation. The results showed the rate of growth to be slow at 30°, rapid at 34° and at 38°, and very slow at 42°.

In a third test, 2-year-old seedling Persian walnut trees growing in cans of sand were placed in temperature-control chambers (3) maintained at 30° and 38°C. Inoculations were made by cutting into the trunk of the trees and inserting bits of agar covered by mycelium of the pathogen. The cuts were covered with waxed paper and wrapped with tape to prevent desiccation of the inoculum. After 2 weeks, the aver-

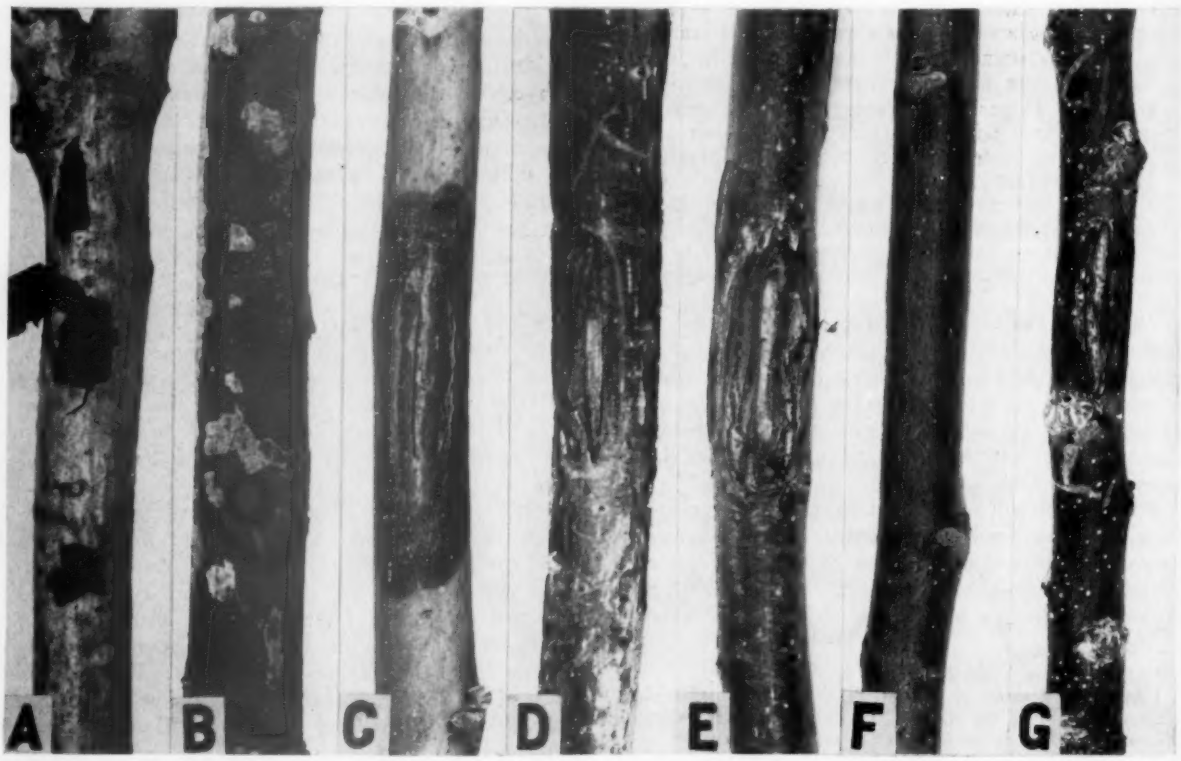

FIG. 1. Persian walnut branches. A) Diseased branch with the phellem beginning to break away to expose the arthrospores of *Hendersonula toruloidea*. B) Diseased branch with the phellem broken away and most of the spores gone. C) Mildly sunburned branch, 1 week after inoculation, showing the initiation of a branch-wilt canker. D) nonsunburned inoculated branch showed no canker development after 2 months. E) Nonsunburned inoculated branch, nearly healed in 1 year. F) Sunburn exposed, noninoculated branch shows very little visible sunburn injury but is susceptible to infection. G) Inoculated branch exposed to sunburn but protected from burn by whitewash showed only minor canker development 2 months after inoculation.

age length of cankers was 9.4 in. at 38° and 6.7 in. at 30°, a difference that is significant at 19:1 odds. Six of the 7 trees held at 38° were completely girdled and were dead, whereas all 7 trees in the 30° chamber were alive.

When the fungus isolate used in these tests was grown on potato-dextrose agar, the optimum temperature for mycelial growth was 30–33°, which agrees with results reported by Wilson (4). At 42° the mycelial extension was slow, suggesting that this temperature is near the maximum for growth of the fungus. The most rapid disease development, therefore, evidently occurs when the temperature is above the optimum for growth of the fungus in artificial media.

The better growth of the pathogen at high temperatures might account for the marked differences in disease severity noted in various walnut growing areas of California. In cool coastal areas the pathogen rarely is found. Even in the hot interior valleys the disease varies considerably in severity. It causes the most damage in the hotter southern part of the San Joaquin Valley (Tulare County) and in the hotter central and northern parts of the Sacramento Valley (Colusa and Tehama counties). In the region at the junction of the 2 valleys (Yolo, Contra Costa, and San Joaquin counties) the fungus is present, but the disease has not been important. These counties lie just east of San Francisco Bay and temperatures are influenced by the cooling breezes from the ocean. The average number of hours above 90°F at selected points in the interior valleys is given in Figure 2.

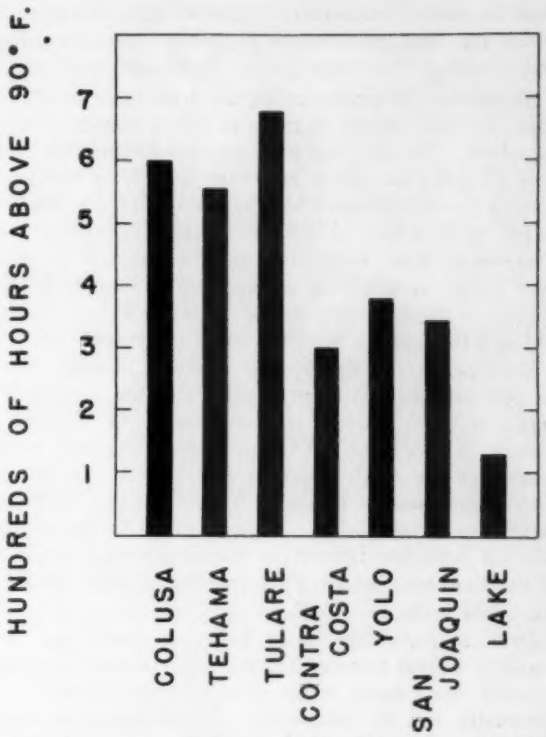

FIG. 2. Yearly average accumulated hours above 90°F in certain walnut growing counties of California. Branch-wilt damage has been severe in Colusa, Tehama, and Tulare Counties but mild in the remaining counties. Temperature data for years 1948-1953 inclusive were obtained from records of the Department of Pomology, University of California, Davis, California.

RELATION OF RELATIVE HUMIDITY TO SPORE GERMINATION.—The effect of relative humidity upon spore germination at the optimum temperature (30°C) for spore germination was studied. Various relative humidity percentages were obtained by placing the spores above different concentrations of sulfuric acid in test tubes, as suggested by Wilson (8). Distilled water was used to obtain a relative humidity approaching 100 per cent.

Forty-five ml of solution was placed in each test tube and the test tubes inserted in a wire rack submerged in a constant temperature water bath to within ¾ in. of the top of the tubes. After 4 hours, when the humidity had reached an equilibrium, the spores were introduced. The spores were lightly dusted on small glass cover slips that were inserted into the bottom of cork stoppers. When the stoppers were placed in the test tubes, the spores were held slightly above the surface of the solution.

After 24 hours, the stoppers were removed and each cover slip was placed on a drop of lactophenol plus cotton blue on a microscope slide. Four spore-bearing cover slips were maintained at each humidity, and 200 spores on each cover slip were counted at random to determine the percentage of germination. Germination occurred at relative humidities above 90 per cent, but not at 90 per cent or below.

POSSIBLE MODE OF INFECTION.—Wilson (4, 6) noted that cankers often center around injuries such as pruning wounds and sunburn injuries. Normally very little, if any, systematic pruning is practiced in walnut orchards although infected branches are removed during the winter. Occasionally, when this is done, a canker develops the following summer, growing back from the location of the cut. It is not clear whether these cankers develop from new infections through the cut surface or from infected tissue that was not removed.

Since cankers frequently develop around areas of bark injured by sunburn, this association is believed to account for the fact that the incidence of the disease is sometimes 2 or 3 times higher on the south than on the north side of the tree (4). The varieties Mayette and Franquette, in which the highest disease incidence has been observed, are particularly subject to sunburn. With both varieties, the limbs grow rapidly in length but produce relatively few side branches. Consequently, the long flexible limbs are scantily protected by the foliage of side branches. The weight of heavy crops tends to bend down the limbs, exposing large expanses of bark to sunburning. Scaffold branches weighted down by a heavy crop occasionally break at the crotch. When this occurs in a limb located on the south or west side, many of the remaining limbs

may be badly sunburned. Harvesting operations in which the limbs are shaken also cause some bruising and breakage that may provide infection courts.

Sunburn.—To ascertain the most common infection sites, a large number of recently killed branches were examined. In the orchards inspected, over 90 per cent of these evidently had been killed by renewed activity of old cankers; however, 60 cankers of recent origin were found. Fifty-five of the cankers of the latter type were associated with a sunburn injury. One killed branch had a mechanical wound, 1 appeared to have been infected through a side shoot, and in 3 the point of infection could not be determined.

Branches killed during the summer of 1953 were counted and their location in relation to the east, west, north, or south sides of the tree was noted. Approximately 75 per cent of the diseased branches were located on the south and west sides of the tree where sunburning would be most severe. This confirms earlier observations by Wilson (4). Furthermore, affected branches located on the north or east sides of the tree were usually in a position where sunburning could occur.

Dead twigs.—Many dead twigs or spurs may be found in walnut trees of bearing age. If these become infected, the fungus might grow into the branch and eventually kill it. Although no sporulation of this fungus was found on dead twigs, 90 were collected at random from infected trees and isolations made. In no case was the branch-wilt fungus recovered. These results are in agreement with earlier work by Wilson (4) who reported that while a *Dothiorella* sp. was frequently isolated, *H. toruloidea* was not.

Bruises.—To simulate the type of injury produced by harvesting operations, branches were bruised by a small mallet. Dry arthrospores were dusted into slits in the bark of the bruised areas of 25 branches during November. The bruises were all healing when examined the following summer and there was no evidence that infection had occurred.

Cuts.—The susceptibility of knife wounds to infection was tested at various times of the year. Immediately after the wounds were made, spores were placed in contact with both xylem and bark tissue. Sixteen branches inoculated during November and another 16 inoculated in March were well healed with no visible evidence of infection when examined the following August (Fig. 1, D, E).

Trees severely damaged by past activity of the fungus were inoculated in knife cuts during the summer of 1954. Sixteen branches were inoculated on each of the following dates: May 1, June 23, and July 14. Half the inoculations were covered with adhesive tape to prevent drying. When the trees were examined September 19, no canker development had resulted from inoculations made May 1 or June 23, although in some cases a necrosis of about ½ in. extended around the inoculation wound from which the fungus could be recovered. Of the 16 branches inoculated July 14, 9 were dead by September 19. These branches apparently had suffered from sunburn, however, since nearby noninoculated and noninfected branches were unmistakably sunburned.

Leaf scars.—To test the possibility that infection might occur through leaf scars, walnut shoots were submerged in a suspension of arthrospores and the leaves were removed while submerged, thus exposing the leaf scars. Half the shoots with inoculated leaf scars were wrapped with tape to minimize desiccation. Of 16 leaf scars inoculated during August, 2 of the 8 taped inoculations resulted in a limited necrosis of host tissue and sporulation of the fungus, but large cankers did not develop. A similar set of 18 leaf scars was inoculated in October during normal leaf fall. When examined the following summer, no infection was evident.

Susceptibility of sunburned branches.—To determine the effect of sunburning on infection and subsequent growth of the pathogen, branches on the south side of trees were bent down and tied in place on July 18, 1953. One month later, each of 19 branches was inoculated at 4 places along the side exposed to the sun. A portion of the periderm was removed with a knife and dry arthrospores were dusted over the wound. Two of the inoculated wounds on each branch were first covered with waxed paper and then wrapped with adhesive tape. The other 2 remained uncovered. For comparison, 19 branches that had not been exposed to the sun were inoculated in a similar manner, and 19 branches that had been exposed were left noninoculated.

On noninoculated branches exposed to the sun from mid-July until mid-October, sunburning could be detected only by close inspection (Fig. 1, F). It consisted of a narrow ribbon of necrotic tissue extending along the upper side of the branch. The branch was not damaged otherwise. Similar inoculated branches, however, developed typical cankers around 36 of the 38 covered wounds, and a like number of cankers developed around the uncovered inoculated wounds. Four of the 19 inoculated sunburned branches were killed by the fungus. On the other hand, no cankers developed on inoculated branches that had not been exposed to sunburn.

A similar test was made during the summer of 1954 in trees that had suffered severe branch-wilt damage in the past. Branches on the south side of the trees were bent down and tied in place June 24. On July 14, very little sunburn was apparent on these branches, but 1 branch bore on its upper surface a small, moist, darkened area that was infested by flat-headed borers, probably larvae of *Chrysobothris mali* Horn. The necrotic area resembled the early stages of branch-wilt, and isolations from all portions of the necrotic area yielded *H. toruloidea.* Ten of these mildly sunburned branches were inoculated by cutting a small slit and inserting dry arthrospores beneath the exposed bark. Ten similarly exposed but noninoculated branches served as the control. When examined September 19, all inoculated branches had large typical

branch-wilt cankers and 9 of the 10 were dead. Two of the noninoculated control branches, moreover, had become infected and were dead.

Scorched branches.—According to Wilson (7), infection of the branch is more readily obtained if, instead of cuts, the inoculum is inserted into bark slightly damaged by heat. Accordingly, small areas of the bark on a number of branches were scorched with a blow torch on August 3, 1953. Ten days later the periderm over these scorched areas was broken and arthrospores dusted into the breaks. Sixty-nine per cent of the 235 inoculated areas became infected. Other branches scorched on August 3 were inoculated in a similar way on November 28, 1953, and March 9, 1954. None of these inoculations resulted in canker development.

Old sunburned areas.—Sunburn-damaged branches are common on walnut trees in the interior valleys of California. The bark over the damaged areas usually sloughs off and exposes the wood. Infestation of these areas by wood-boring insects is common, and active cankers of the branch-wilt disease sometimes develop around them. In many cases, however, they show no visible signs of the fungus. Yet isolations revealed that the fungus was present in 23 per cent of the old sunburned areas on trees in 1 badly diseased orchard. Possibly the fungus was either quiescent or existed as a saprophyte in old sunburn-damaged wood without immediate invasion of adjacent living tissue.

Will the fungus, which has been quiescent or growing as a saprophyte in dead tissues of a canker, invade the adjacent live tissue if the branch is exposed to the sun? An experiment was devised to test this possibility. Certain branches had been inoculated the previous year, but the fungus had made only a limited growth and the inoculation wounds had healed. Five of the branches were exposed to the sun on June 15. By August 15, all 5 had been killed by

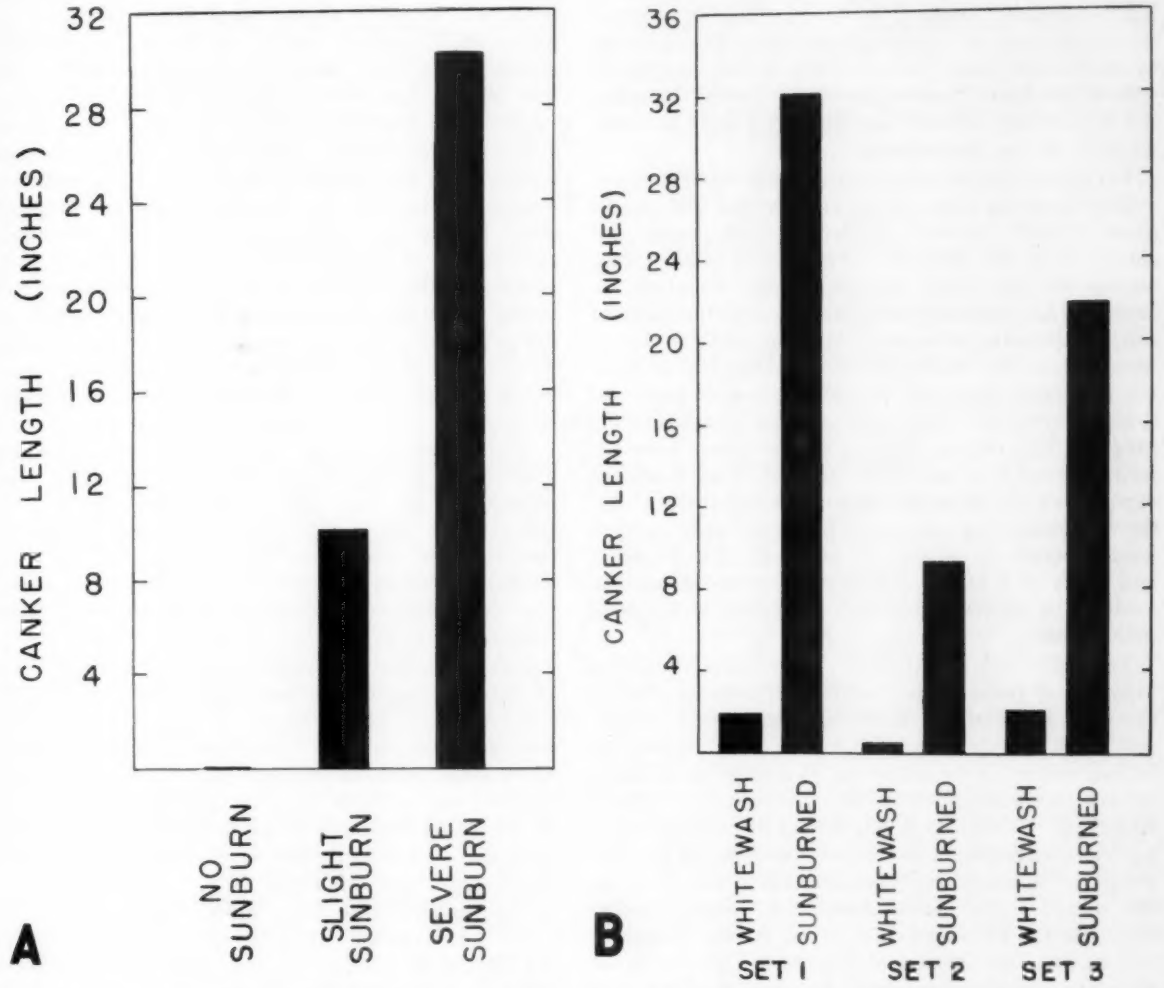

FIG. 3. The effect of sunburn upon subsequent branch-wilt canker development. A) Length of cankers 1 month after inoculation in branches severly sunburned, slightly sunburned, and nonsunburned. The greater the sunburn injury, the longer the canker. B) Results of 3 sets of inoculations made to test the effect of whitewashing on canker development in walnut branches.

well defined branch-wilt cankers that developed around the inoculation scars. No cankers developed in noninoculated branches that had been exposed to the sun at the same time.

In a second test, old sunburn areas were inoculated, but the branches were not later exposed to fresh sunburning of adjacent healthy tissues. Spores were inserted into the dead wood and adjoining healthy tissue of 16 branches. In 1 year, however, no parasitism of surrounding healthy tissue occurred.

EFFECT OF SEVERITY OF SUNBURN DAMAGE UPON CANKER DEVELOPMENT.—The rate of canker extension was followed in inoculated branches that had been subjected to varying amounts of sunburn damage. After exposure and inoculation, the tied-down branches were released to reduce subsequent sunburn. One month later, the cankers resulting from inoculation of necrotic tissue in severe sunburned areas were, on the average, nearly 3 times longer than cankers resulting from inoculation of exposed but nonnecrotic areas (Fig. 3, A). Yet, in spite of the slower rate of canker development, the majority of mildly sunburned branches were killed by branch-wilt in 30 days. Noninoculated, sunburned branches did not develop cankers, nor did they appear seriously affected by the sunburning.

EFFECTIVENESS OF WHITEWASH IN DISEASE REDUCTION.—The foregoing experiments indicate that the fungus grows rapidly through sunburned bark tissue and slowly, if at all, through nonsunburned tissue. Protecting the tree from sunburn should, therefore, be beneficial in preventing infection. To test this hypothesis, comparisons were made between canker development in branches protected from sunburn and in those not protected. On June 16, 1954, lateral branches of 2 walnut trees were bent over and tied to stakes in the ground. The central leaders of the trees were too large to bend over and were removed. This treatment exposed all the branches to sun throughout the day, thereby subjecting the trees to more sunburn than usually occurs in commercial orchards. The branches and trunk of 1 of the 2 trees were protected against sunburn by whitewashing with a mixture of hydrated lime in water.

Two sets of inoculations were made into the branches of these 2 trees in areas of bark exposed to the sun. The first set of 16 inoculations in each tree was made on July 8 by splitting the periderm and inserting spores. A second set of 24 similar inoculations in each tree was made July 19. Results were recorded August 17, 1954 (Fig. 3, B). In the first set, the average canker length for the whitewashed tree was 2 in., compared to 32 in. for nonwhitewashed branches. In the second set of inoculations, the average canker length in the whitewashed tree was 0.5 in., compared to 9 in. for nonwhitewashed branches. Eighteen of the 43 nonwhitewashed inoculated branches died, but only 1 of the 38 whitewashed inoculated branches was killed. The 1 dead branch in the whitewashed tree and 1 of the dead branches in the nonwhitewashed tree had been girdled 3 years before and were evidently in a weakened condition before the experiment began.

A third set of inoculations was made in branches of 2 trees exposed to sunburn on June 30. The branches of 1 tree were immediately whitewashed, whereas the branches of the second remained unprotected. Seventy-five inoculations were made in the branches of each tree on July 19. By September 1, the average canker on the whitewashed tree was 2 in. long, and on the nonwhitewashed tree, 22 in. long (Fig. 3, B). None of the 37 branches on the whitewashed tree were killed, whereas 25 of the 41 inoculated branches of the nonwhitewashed tree were killed. Such differences were obtained even though the whitewash, in a number of cases, had flaked off badly before final records were taken. Cankers on whitewashed branches almost always occurred where the whitewash was largely gone (Fig. 1, G). On the nonwhitewashed tree the branches were about equally distributed around the tree. It was possible, therefore, to compare the rate of canker development on the south and north sides. The average length of 27 cankers on the north side was 15 in., whereas the average length of 27 cankers on the south was 30 in. The few cankers developing on whitewashed trees were all on the south side.

DISCUSSION.—Young, vigorously growing Persian walnut trees are seldom damaged by the branch-wilt disease. At bearing age, however, limbs are weighted down by the crop and subjected to sunburn which causes them to become more susceptible. Sunburn is common in the orchards of the hot central valleys and occurs when temperature conditions are favorable to the growth of the causal fungus. It not only provides infection courts but, in addition, predisposes the tissue to subsequent invasion. Wounds, such as those made by pruning and harvesting tools, evidently do not greatly influence the susceptibility of nonsunburned branches. Freezing injury has been associated with infection by *H. toruloidea* in citrus orchards (2), but this type of injury rarely occurs on walnuts in the central valleys and was not encountered in these studies. Moreover, recent orchard observations suggest that sunburn is important in predisposing citrus trees to infection (1).

Once branches have been predisposed to infection by sunburn, apparently any rupture of the periderm will allow the fungus spores to gain access to susceptible tissues below. The periderm may be broken when scratches occur as a result of branches rubbing together during windy days. In addition, ruptures may be caused by the wood-boring larvae of certain insects such as *Chrysobothris mali* Horn, which are quick to attack sunburned branches.

High relative humidities were found necessary for spore germination. While the atmosphere is usually dry during the summer period, necessary moisture is evidently provided by host tissue. Since spores placed beneath the bark of nonsunburned branches also germinate, failure to develop cankers in the latter case apparently is not due to failure of spore germination.

Old sunburn lesions, beneath which wood has been dead for several years, frequently become infested by *H. toruloidea.* Since in such lesions there is no evident invasion of adjacent living tissues, it is believed the fungus grows saprophytically through the dead wood. Results of tests reported herein, however, indicate that the fungus in such branches may become actively parasitic if the branches are subsequently exposed to additional sunburn.

The prevention of disease by the application of whitewash to reduce sunburn suggests a possible control measure. It has not been determined, however, if the application of whitewash to commercial orchards would provide protection long enough to effect control. Nevertheless, any orchard practice that reduces sunburn damage can be expected to reduce the severity of the disease.

Before the appearance of branch-wilt, sunburn was not regarded as a serious problem of walnut production in the central valleys of California. With the wilt fungus present, however, sunburn becomes of major importance.

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RELATIONSHIP OF XANTHOMONAS PELARGONII AND XANTHOMONAS GERANII [1]

Mortimer P. Starr, Zafrira Volcani[2], and Donald E. Munnecke

SUMMARY

By means of comparative biochemical and cultural determinations and cross-inoculation tests with authentic cultures of *Xanthomonas pelargonii* (Brown) Starr & Burk. and *Xanthomonas geranii* (Burk.) Dowson, the 2 species were shown to be identical. It is recommended that the 2 species be combined in *Xanthomonas pelargonii.*

The bacterial stem and leaf disease of *Pelargonium hortorum* (commonly called geranium) caused by *Xanthomonas pelargonii* (Brown) Starr & Burk. has limited the production of cuttings from field-grown geraniums in California. Earlier reports by Hellmers (6) and Munnecke (7) describe the disease as it occurs in Denmark and in California, clarify the relationship between the leaf and stem phases of the infection, and outline practicable methods of control. The relationship of the Pelargonium disease and its causal agent to the similar disease of *Geranium* species and its causal agent, *Xanthomonas geranii* (Burk.) Dowson (2), deserves further examination. Re-evaluation of this relationship is desirable because Burkholder (1), noting that "only a guess could be made on the relationship of the two" bacterial species and that "the geranium leaf-spot bacterium does not appear to be pathogenic on Pelargonium," had concluded that the geranium pathogen was a previously undescribed species. Accordingly, a comparative bacteriological and phytopathological study was made of authentic cultures of the 2 species.

Bacteriological study.—*Cultures.*—A representative collection of 13 cultures of *X. pelargonii* isolated from *Pelargonium hortorum* and 4 of *X. geranii* isolated from *Geranium* species was assembled[3]. These were checked for purity by repeated plating from

[1] Accepted for publication July 13, 1955.
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[3] The cultures of *X. pelargonii* were isolated by W. H. Burkholder, E. Hellmers, C. Perrault, K. F. Baker, and D. E. Munnecke, 1938 to 1952. Those of *X. geranii* were isolated by W. H. Burkholder in 1935 to 1940. All cultures had been preserved by lyophilization, starting in 1939.

dilute aqueous suspensions on yeast-glucose-$CaCO_3$ agar. Each culture was checked for virulence on its homologous host.

Methods.—Pure cultures that were virulent were subjected to various cultural and biochemical determinative tests by methods that have been detailed in other publications (9).

Results.—The morphological and physiological reactions of the 2 species were generally identical. The rare deviations from the "typical" reaction occurred as frequently among different isolates of the same species as between the 2 species. These differences were mainly of degree; e.g. the time required for complete liquefaction of gelatin, for forming acid from sugars, or for producing an alkaline reaction from organic acids. The morphology, staining reactions, and cultural characteristics of the 17 cultures were essentially the same as those described by Hellmers (6) for *X. pelargonii*. In the following summary of our observations, the description applies to all 17 cultures, including both *X. pelargonii* and *X. geranii*, except where stated otherwise.

In litmus milk, a soft curd appeared within a week, followed by peptonization.

Nitrite was not detected in nitrate broth cultures.

Except for 2 strains of *X. pelargonii*, all cultures liquified nutrient gelatin within 2–3 weeks.

Hydrogen sulfide was not detected in lead acetate agar cultures, but blackening of lead acetate paper suspended over cultures in various media gave evidence of its presence.

Starch was not hydrolyzed, as shown by the iodine test of cultures on nutrient starch agar.

Formation of red color, followed by browning, was demonstrated in the tyrosine medium of Burkholder and Starr (3), indicating a positive "tyrosinase" reaction.

Voges-Proskauer and methyl red tests were negative.

Slight lipolytic activity was demonstrable on plates of spirit-blue cottonseed-oil agar (10).

Slight pectinolytic ability was shown by slow liquefaction of calcium pectate stab cultures (3, 9).

In a simple ammonium-glucose-salts solution, growth from small inocula was fair to good when the procedure described by Starr (8) was used.

In the "synthetic" peptone-free basal medium of the Manual of Methods (4), all cultures produced acid but not gas from glucose, galactose, mannose, xylose, lactose, sucrose, and glycerol. A total of 4 cultures (2 of each species) slowly formed acid from mannitol. No acid was formed from maltose, sorbitol, salicin, or dextrin.

Ability to utilize salts of various organic acids was tested by 2 procedures. In 1 method, the neutralized acid (0.15 per cent) was added to tubes of the peptone-free liquid basal medium used in the carbohydrate fermentation tests. Since this concentration of certain organic acids was shown by suitable control experiments to inhibit growth, a gradient-plate method (11) also was tried. A layer of nonnutrient plain agar was allowed to harden in a Petri dish held in a slanted position. Subsequently, a layer of nutrient agar (basal medium plus 0.3 per cent neutralized organic acid and 1.5 per cent agar, pH 6.9) was poured over the slanted plain agar and allowed to harden with the Petri dish held horizontally. The downward diffusion of the acid in the top layer provided a graded series of concentrations of the acid. The cultures were streaked in straight lines along the gradient so that the cells were exposed to a range of concentrations of each acid. All cultures grew and produced alkaline reactions on acetic, citric, formic, lactic, malic, malonic, and pyruvic acids; however, growth on formic, lactic, and pyruvic acids was not as heavy as in the others. Hippuric and tartaric acids were not utilized.

CROSS-INOCULATION TRIALS. — Since *Xanthomonas pelargonii* and *X. geranii* are indistinguishable by bacteriological tests, it was of interest to examine the pathogenicity of the 2 species by cross-inoculation of the respective host plants. Two horticultural varieties of *Pelargonium hortorum* Bailey, Radio Red (extremely susceptible) and Red Fiat (moderately susceptible), and 5 species of *Geranium* (*G. maculatum* L., *G. pratense* L., *G. sanguineum* L., *G. sylvaticum* L., and *G. yedoënse* Franch. & Sav. were used. *P. peltatum* Ait. (Ivy geranium) was used in 1 test. The identification of the species of *Geranium* was verified by Dr. Mildred Mathias, Department of Botany, University of California, Los Angeles. All plants were inoculated by spraying the leaves with a suspension of bacteria from 36- to 48-hour cultures in beef-peptone broth. This was followed by incubation in a moist chamber for 24 hours. The lids of the moist chambers were removed and the plants allowed to dry for 24 hours before they were returned to the greenhouse bench. In test number 1 (Table 1), additional plants of *P. hortorum* were inoculated by stem puncture and replaced in the greenhouse without incubation in the moist chamber. Cross-contamination of the bacterial cultures was prevented by several precautions: atomizers were sterilized between inoculations; plants were inoculated in separate incubators for each isolate; hands were washed thoroughly between operations; plants were widely spaced in the greenhouse, and the foliage was kept dry. Noninoculated control plants were handled in the same manner except that they were sprayed with sterilized beef-peptone broth.

The results of 2 inoculation tests are shown in Table 1. Unfortunately, results of inoculation test 1 were obscured by the onset of hot dry weather that caused the leaves of the *Geranium* species to wither and dry shortly after removal from the incubators, but very definite indications of pathogenicity were apparent on the varieties of *P. hortorum*, which were unaffected by the environmental conditions. The most virulent

TABLE 1.—*Cross-inoculation tests of Xanthomonas geranii and X. pelargonii*[a]

Isolates	Reaction on indicated host plants							
	Pelargonium hortorum		*Geranium*					*Pelargonium peltatum*
	Radio Red	Red Fiat	*G. maculatum*	*G. pratense*	*G. sanguineum*	*G. sylvaticum*	*G. yedoënse*	
			Inoculation test 1[b]					
X. pelargonii								
754	++, R	+	?	+, R	?	?	?	
781	++	+	+, R	?	?	?	?	
G29	+++, R	+++	+, R	+, R	?	?	?	
X. geranii								
XG1	+	−	?	?	?	?	?	
XG4	+	+	+, R	?	?	?	?	
XG6	?	−	?	?	?	?	?	
783	+, R	−	+, R	?	?	?	+, R	
			Inoculation test 2[c]					
X. pelargonii								
G29 (RR)	+++		++	+	+	+	+	+
G29 (Mac)	++		++	+			−	++
G29 (Prat)	+		+	+			−	+
781	?		−	+			−	+
X. geranii								
XG4 (Mac)	+		+	+	++	+	−	+
XG1 (RR)	+		+	?			−	+

[a] +++, plants killed; ++, stem rot visible (*P. hortorum*) or abundant leaf spots; +, few leaf spots or angular necrosis; −, not infected; ?, doubtful reaction.

[b] Symptoms obscure because of poor host growth of *Geranium* spp. R indicates that bacteria were isolated from leaves. These isolates were pathogenic to *P. hortorum*.

[c] Bacterial cultures were reisolates from plants inoculated in the first test. The source plants are indicated by the following designations: RR = Radio Red, Prat = *G. pratense*, and Mac = *G. maculatum*.

isolate was G29 (*X. pelargonii*), a fresh isolate, whereas culture XG6 (*X. geranii*) was avirulent, although 1 Radio Red plant inoculated by stem puncture appeared to be slightly infected. The reaction of the Radio Red variety showed that the 2 species of bacteria were capable of infecting *P. hortorum*, but the difficulty of distinguishing leaf injury on the *Geranium* species grown under hot dry conditions from symptoms of bacterial infection left the status of the reaction of *Geranium* species in doubt.

Dilution plates were made of chopped leaves and petioles of all of the plants that showed leaf-spot symptoms as well as those from the noninoculated control plants. Thirteen cultures with yellow colonies were isolated including 3 from noninoculated controls. All of these were screened for pathogenicity by stem-puncture inoculation of young Radio Red plants. None of the isolates from the controls were pathogenic, whereas both species of bacteria were obtained from both host genera in the inoculated series. Thus, *X. pelargonii* was isolated from Radio Red, *G. maculatum*, and *G. pratense;* and *X. geranii* was isolated from Radio Red, *G. maculatum*, and *G. yedoënse*. Probably more of the species of Geranium were infected by the bacteria, but since it was impossible to isolate the bacteria from them, the data did not warrant the conclusion that these species were infected.

In the second inoculation test (Table 1), the inocula consisted of reisolates of 1) *X. pelargonii* from Pelargonium, 2) *X. pelargonii* from Geranium, 3) *X. geranii* from Pelargonium, and 4) *X. geranii* from Geranium. In addition to the 5 species of *Geranium* and the Radio Red variety of *P. hortorum* that were used in the first test, *P. peltatum* (Ivy geranium) was included. The plants were inoculated by spraying bacteria over the leaves. They were treated as described for the first test, except that the pots were set in wood shavings in the greenhouse benches. This decreased loss of water from the pots, and the leaves of the *Geranium* species did not dry out as much as in the first test. This second test clearly showed that the 2 species of bacteria may infect *P. hortorum*, *P. peltatum*, and all of the *Geranium* species, although *G. yedoënse* was only slightly susceptible.

The symptoms produced by the 2 species of bacteria were identical, except that the various isolates varied somewhat in virulence. The fresh isolate G29 of *X. pelargonii* was the most virulent whereas a few of the older isolates were low in virulence. One isolate, XG6 of *X. geranii*, was practically avirulent. The tendency of *X. pelargonii* to lose virulence in culture is common, and in other experiments with this species it was found necessary to use fresh isolates for maximum virulence. The symptoms of the 2 bacteria on *P. hortorum* depend upon the variety used as well as the method of inoculation. If the stem only is inoculated and care is taken to prevent contamination of leaves, infection is initially systemic and results in typical vascular discoloration. The leaves

wilt, become extremely flaccid, and abscise. Eventually the stems blacken and rot, and the plant may die. Frequently, angular necrotic areas appear on the leaves where symptoms apparently are restricted to the area between the main veins. When stems are inoculated in this manner, the leaf-spot stage of the disease is not evident.

If the bacteria are sprayed over the leaf surface, 2 symptoms may be noted. On most varieties, such as Radio Red, the initial symptoms are typical leaf-

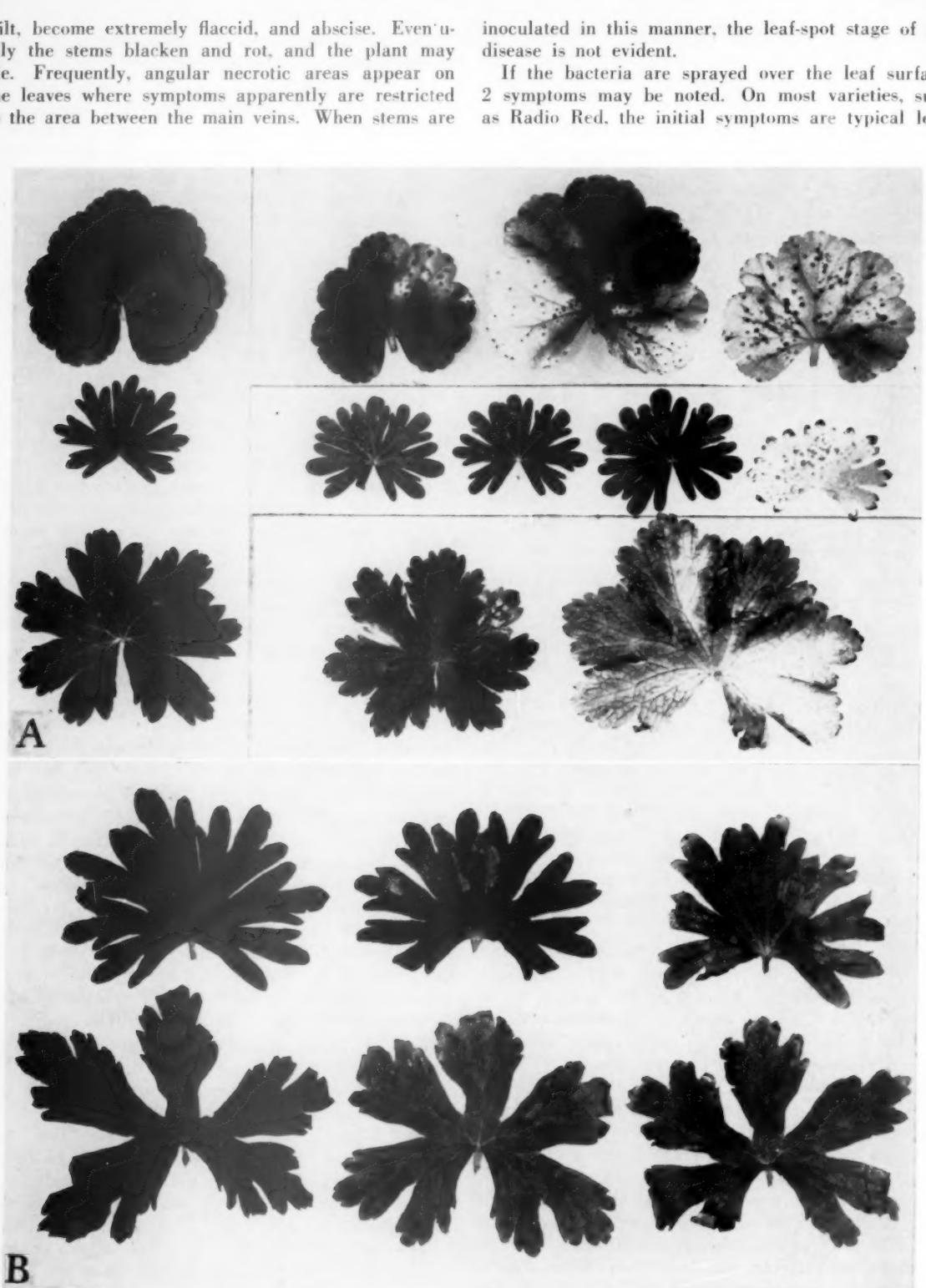

FIG. 1. A) *Pelargonium hortorum* (top), *Geranium sanguineum* (middle) and *G. sylvaticum* (bottom) inoculated with *Xanthomonas pelargonii*, culture G29, reisolated from Radio Red variety of *P. hortorum*. Noninoculated control on left. B) *Geranium sanguineum* (top) and *G. maculatum* (bottom) inoculated with *Xanthomonas geranii*, culture XG4, reisolated from *G. maculatum*. Noninoculated control on left. Photos by B. B. Markley.

spot symptoms, consisting of small watersoaked spots. Within 2–3 days after the spots are apparent, the leaves become yellow and flaccid and may fall off. At this stage the spots are necrotic and have a hard, dry appearance, and bacteria are present in large number in the tissues.

These stages in the development from young leaf-spot infections to the advanced necrotic stage on *P. hortorum* are shown in Figure 1, A. The bacteria later penetrate the stem and cause the typical stem-rot phase. On other varieties, such as Red Fiat, the leaf-spot stage does not occur; instead the angular necrotic pattern described above develops. Eventually, such plants may succumb to the stem-rot phase.

The symptoms of the disease on the *Geranium* species were essentially as described by Burkholder (2). Small spots 2–3 mm in diameter were common on *G. sanguineum*, whereas extreme tip necrosis and general leaf chlorosis was common on *G. maculatum* (Figure 1, A, B). The spots tended to coalesce into irregular necrotic areas of the leaf. All of the severely infected leaves tended to become yellow and to die; occasionally the leaves of *G. sanguineum* reddened prior to abscision. No crown or systemic infection was noted in these experiments, and none of the plants were killed by the inoculations.

Discussion and conclusion.—Our description of the group consisting of *X. geranii* and *X. pelargonii* is in substantial agreement with the description given by Burkholder (2) for *X. geranii* and by Hellmers (6) for *X. pelargonii*. The occasional differences in results reported by the several laboratories may in part be due to different experimental methods and interpretations. Other possible sources of confusion are noted by Hellmers (6, p. 26-27). The serological homogeneity of the 2 "species" has been reported by Elrod and Braun (5).

The original proposal for naming *X. geranii* as a new species, different from *X. pelargonii*, stemmed from Burkholder's inability to obtain infection of the "common red-flowering variety" of *Pelargonium hortorum* with cultures of *X. geranii*. A possible explanation of his results is suggested by the observations reported herein on the failure of resistant horticultural varieties of *P. hortorum* to develop typical leaf-spot symptoms when inoculated with *X. geranii*. If the variety used by Burkholder was a resistant one like some of those used in the present studies, the leaf-spot phase would not have appeared and other evidences of infection may have been overlooked.

In view of the bacteriological, phytopathological, and serological evidence for synonomy of the 2 species, it is recommended that *Xanthomonas geranii* (Burk.) Dowson and *Xanthomonas pelargonii* (Brown) Starr & Burk. be merged into the single species, *Xanthomonas pelargonii* (Brown) Starr & Burk. *X. pelargonii* was named (1) in 1923 and *X. geranii* in 1937, hence the former name has priority.

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INHIBITION OF CROWN GALL BY SELECTED ANTIBIOTICS [1]

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SUMMARY

Eight antibiotics were tested for their effects on 1) growth *in vitro* of the crown gall bacterium, 2) development of crown gall overgrowths in plants, and 3) growth and appearance of normal plant tissues. Tomato and marigold were used as test plants.

Growth of 3 strains of *Agrobacterium tumefaciens* in modified medium 79 was inhibited at the 50 per cent level by aureomycin, terramycin, and chloramphenicol within a range of 0.026 to 0.28 μg/ml. Inhibition at this level by the other antibiotics occurred between 2.05 and 8.4 μg/ml. The variation in sensitivity of the 3 strains used was not significant.

All antibiotics were introduced into the test plants by root absorption. Crown gall development in tomato plants inoculated with a virulent strain of *A. tumefaciens* was inhibited at the 50 per cent level by 2 μg/ml of terramycin, 5 μg/ml of aureomycin, and 4 μg/ml of chloramphenicol. Less effective were streptomycin, clavacin, polymyxin, penicillin, and tyrothricin.

Terramycin, aureomycin, and chloramphenicol showed little or no toxicity to normal plant tissue at concentrations of 20, 50, and 125 μg/ml, respectively, yet these levels caused complete gall inhibition. At a concentration of 500 μg/ml, which caused nearly complete gall inhibition, streptomycin was moderately phytotoxic. Clavacin at 60 μg/ml, polymyxin at 750 μg/ml, tyrothricin at 1500 μg/ml, and penicillin at 2000 μg/ml differed little in toxicity to normal and gall tissue.

A culture of *A. tumefaciens* resistant to chloramphenicol and another resistant to polymyxin were used in the inoculation of antibiotic-treated plants. Also, bacteria-free crown gall tissue was grown on a medium containing either chloramphenicol, polymyxin, or terramycin. All 3 antibiotics caused inhibition of the bacteria-free gall tissue. Chloramphenicol and polymyxin, tested in plants inoculated with resistant bacteria, caused similar inhibition of gall development. These 2 antibiotics induced a distinct type of gall inhibition curve in these 2 tests, as well as during inhibition of galls in plants inoculated with antibiotic-sensitive bacteria. This sameness in inhibition pattern may indicate a common type of action in tissue culture and in plants. Terramycin induced a different inhibition response in gall tissue cultures than in plants and thus may have more than 1 type of action.

The evidence suggests that gall inhibition by aureomycin, terramycin, and chloramphenicol was caused, in large part, by antibiotic activity against the crown gall cells. This action in the gall tissue was accompanied by little or no injury to the normal plant cells.

Terramycin and chloramphenicol absorbed by the roots of tomato plants moved rapidly into the leaves. Concentrations of terramycin were equal in the roots and leaves. Little of either antibiotic remained in the stems. After 10 days terramycin and chloramphenicol were 47 and 80 per cent active, respectively, in the leaf tissue.

Various aspects of antibiotic usage as plant chemotherapeutic agents appear in recent reviews (6, 7, 14, 25, 35, 38). Since the contributions of Brown (9) and Brown and Boyle (10, 11), the potential effectiveness of antibiotics in the control of crown gall caused by *Agrobacterium tumefaciens* (E. F. Sm. & Town.) Conn has become increasingly apparent.

Growth *in vitro* of *A. tumefaciens* is readily inhibited by various antibiotics. Waksman et al (36) and Gilliver (20) noted inhibition by 14 substances including crude extracts and highly purified preparations of penicillin, streptomycin, streptothricin, clavacin, tyrothricin, and actinomycin. De Ropp (13) tested seven purified antibiotics against 6 strains of *A. tumefaciens*. Aureomycin, chloramphenicol, streptomycin, and streptothricin showed the greatest activity.

The destruction or inhibition of experimentally induced crown galls has resulted from various methods of antibiotic application. Brown and Boyle (11) exterminated galls on bryophyllum by wrapping the galls with cotton wool soaked in crude penicillin and then pricking the wrapped galls. Similarly Hampton (21) employed both penicillin and streptomycin in destroying crown gall on bryophyllum, tomato, and other plants. Dye (16) observed that gall formation on marigold was prevented by injecting streptomycin into the stems adjacent to inoculation sites, but the smearing or brushing of this antibiotic over galls gave only negative results. In similar tests (15, 17, 18) negative results were obtained with penicillin, streptothricin, and actinomycin. Blanchard (3) noted almost complete inhibition by aureomycin of galls formed on tomato plants grown in a mineral salts solution containing the antibiotic. De Ropp (13) obtained

[1] Accepted for publication July 11, 1955.

This work was supported in part by the American Cancer Society and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. The authors are indebted to Eugene Herrling for assistance with the illustrations.

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inhibition of gall formation on carrot storage tissue by penicillin and streptomycin; growth *in vitro* of bacteria-free gall tissues was inhibited by penicillin.

The manner in which antibiotics cause inhibition or destruction of crown gall tissue has been suggested by several workers. Brown (9) noted that the nuclei of gall cells were destroyed by concentrations of penicillin and of streptomycin that had no effect on normal tissue. From studies with bacteria-free gall and normal tissue of sunflower, de Ropp (12) concluded that streptomycin acted as an inhibitor of embryonic tissue rather than as a specific inhibitor of crown gall. Although penicillin was more toxic to bacteria-free gall tissue than to normal tissue of tobacco, Rybak (33) concluded that the type of action was identical on both tissues. In contrast to the above findings with bacteria-free gall and normal tissue, the inhibitory action of streptomycin (13) and of aureomycin (3) on the formation of gall tissue has been interpreted as a direct effect on the causal organism.

The ability of various plants to absorb and to translocate certain antibiotics is an important facet of antibiotic treatment. Earlier investigators have described the uptake and translocation of streptomycin by soybeans (2), of aureomycin by lima beans (4), and of griseofulvin by lettuce (8). Pramer (29) demonstrated the uptake and translocation of chloramphenicol and streptomycin by the roots of cucumber seedlings but was unable to show similar uptake by aureomycin, terramycin, or neomycin. Mitchell et al (27) observed rapid absorption and translocation of streptomycin applied in lanolin paste to the stems of bean plants. Robison et al (31) showed that both streptomycin and terramycin were readily absorbed and translocated by chrysanthemum cuttings, but less readily by rooted plants.

The present paper discusses the effects of selected antibiotics on 1) growth *in vitro* of the crown gall bacterium, 2) formation and growth of crown gall tissue, and 3) the health and appearance of normal plant tissue. Data are presented on the translocation and stability of 3 antibiotics in plant tissue.

MATERIALS AND METHODS.—The 8 different antibiotic substances used were aureomycin hydrochloride (chlortetracycline),[4] terramycin hydrochloride (oxytetracycline),[5] chloramphenicol,[6] streptomycin sulfate, polymyxin B sulfate, penicillin G potassium, clavacin, and tyrothricin.

Five strains of *A. tumefaciens* served as test organisms, namely, the virulent T11 and A6 strains (30, 37), the attenuated A6-6 strain (22), and 2 subcultures of the A6 strain, 1 resistant to chloramphenicol and 1 resistant to polymyxin. The first 3 strains were obtained from the local Department of Plant Pathology; the other 2 cultures were developed in the course of this study. Single-cell isolations of each strain of *A. tumefaciens* were made at the beginning of this study. Purity of all cultures was confirmed periodically by plating on aniline blue agar (22). All cultures and subcultures were maintained on medium 79 (1).

Tomato (*Lycopersicon esculentum* Mill. var. Bonny Best) and marigold (*Tagetes erecta* L. var. Sunset Giant) were used as test plants. The plants were grown during all seasons of the year in the greenhouse in 4-in. pots containing 3 parts loam-manure compost and 1 part sand. At the time of treatment the plants were 4–6 weeks from the seed stage or 3–5 weeks from the transplant stage. The average wet weight of the test plants at the time of antibiotic treatment and inoculation was about 20 g; the average height was about 12 in. The antibiotics were administered by immersing the washed roots of the test plants in 10 or 20 ml of the respective aqueous antibiotic dilution. Following uptake of the solution the plants were repotted in fresh soil.

Immediately following each antibiotic treatment the experimental plants were inoculated with *A. tumefaciens*. The inocula were prepared by incubating cultures in medium 79 at 30°C on a rotary shaker at 350 rpm until an optical density reading of 0.5 was obtained with a Coleman Junior Spectrophotometer set at a wave length of 585μ. At the time of plant inoculation, 0.005 ml of a liquid culture was added by means of micropipette to half-spear point punctures made through each of 4 internodes along the main stem.

The assay medium in the *in vitro* tests was medium 79, modified as follows: mannitol, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; yeast extract, 5.0 g; and distilled water, 1 liter. The reaction was pH 6.8–7.0. All cultures were incubated for 6–8 hours on a rotary shaker at 350 rpm at 30°C. The low sugar content of the medium allowed only minimum gum formation.

EFFECT OF THE ANTIBIOTICS ON GROWTH OF A. TUMEFACIENS.—Each of the 8 antibiotics was assayed turbidimetrically against the A6, A6-6, and T11 strains of *A. tumefaciens*. The assay procedure followed the method used by Joslyn and Galbraith (24). The antibiotics were prepared in 4 serial dilutions in sterile distilled water and added in 1-ml amounts to triplicate matched test tubes containing 8.6 ml of assay medium. Control tubes were prepared by adding 1 ml of sterile distilled water, instead of the antibiotic, to the 8.6 ml of assay medium. To each tube was added 0.4 ml of a suspension of the test strain of *A. tumefaciens*. The optical density of the suspension was 0.5, which corresponded to about 1 billion cells per ml. The tubes were then incubated until the turbidity of the control tubes reached an optical density of about 0.5. Optical density readings then were taken on all tubes and the readings of replicate tubes were

[4] Aureomycin is 7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.

[5] Terramycin is 4-dimethylamino-1,4,4a,5,5a,6,11,12a-octohydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.

[6] Chloramphenicol is D(−)-*threo*-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl] acetamide.

averaged. Inhibition percentages were calculated from the equation:

$$\text{Inhibition percentage} = 100 - \left(\frac{a}{b} \times 100\right) \quad \text{I}$$

where a is the averaged optical density of 3 cultures at each antibiotic concentration, and b is the averaged optical density of cultures without antibiotic.

The endpoint of the assay, i.e., the ID 50 value, was the concentration or dose causing 50 per cent inhibition of growth of the test organism. This endpoint was determined by 1) calculating the percentage inhibition of growth at each concentration of antibiotic, and 2) plotting these data against corresponding antibiotic concentrations to obtain an inhibition curve. The final ID 50 values were derived from inhibition curves prepared from 3 replicate assays (Fig. 1).

The ID 50 values for all 8 antibiotics are listed in Table 1. The broad spectrum antibiotics, aureomycin, terramycin, and chloramphenicol, were particularly potent in retarding growth. Streptomycin, clavacin, and polymyxin, which display narrower spectra, were much less effective. Penicillin and tyrothricin, active largely against gram positive organisms, were the least potent.

The 3 strains of *A. tumefaciens* tested varied only slightly in their sensitivity to the 8 antibiotics at the 50 per cent level of inhibition. As antibiotic concentrations increased, however, the curves generally became more accentuated. Accordingly, differences among the strains became more evident. De Ropp (13) found pronounced variation when a 100 per cent level of inhibition was used as the endpoint of a dilution type assay.

THE EFFECT OF ANTIBIOTIC ACTION ON CROWN GALL TISSUES.—Eight antibiotics, absorbed through the roots of the plants, and the antibiotic-sensitive strain of *A. tumefaciens* were used. Triplicate experiments with tomato as the host plant were made with each antibiotic. Terramycin, chloramphenicol, and polymyxin were used in duplicate experiments with the marigold plant. The concentration or dose of antibiotic capable of bringing about 50 per cent inhibition of gall development, i.e., the ED 50 value, was estimated in each case. Concentrations of antibiotic estimated as causing 100 per cent inhibition, or the ED 100 value, were recorded wherever they occurred.

About 30 days after treatment the galls were severed from the stems of replicate plants and weighed. Inhibition percentages for each antibiotic were determined by the modified equation:

$$\text{Gall inhibition percentage} = 100 - \left(\frac{a}{b} \times 100\right) \quad \text{II}$$

where a is the average weight of galls at each dilution of antibiotic, and b is the average weight of galls on the control plants. ED 50 values were obtained from inhibition curves by plotting the averaged percentages for replicate experiments against corresponding antibiotic concentrations (Table 2). Typical inhibition curves for the sensitive A6 strain against chloramphenicol, terramycin, and polymyxin are shown in Figures 2, 3, and 4.

The relatively low ED 50 values for terramycin, aureomycin, chloramphenicol, streptomycin, and clavacin corresponded to marked inhibition of gall growth (Table 2). As judged by ED 100 values, however, only the first 3 of these antibiotics were reasonably effective in causing complete inhibition of gall growth. A comparison of ED 50 and ED 100 values (Table 2) with minimum and lethal phytotoxic levels (Table 3) shows that only these 3 antibiotics were effective as control agents, for complete gall inhibition occurred at levels exerting little or no phytotoxic effects. Streptomycin was moderately effective.

Stimulation of gall development by polymyxin at the lowest level used (12 μg/ml) was indicated. Although gall formation was completely inhibited on the plants treated with terramycin, aureomycin, or chloramphenicol, isolations made from the stems of these plants showed that the causal organisms were present and viable. Whether these bacteria were present in pockets of the dead and suberized tissue near the inoculation punctures where the antibiotics had not penetrated was not determined.

Data on the experiments with terramycin, chlor-

TABLE 1.—*Inhibition dosage (ID 50) values of the 3 test strains of A. tumefaciens*

Antibiotic	Strains of A. tumefaciens[a]		
	A6	T11	A6-6
	μg/ml	μg/ml	μg/ml
Aureomycin	0.032	0.026	0.029
Terramycin	0.036	0.043	0.042
Chloramphenicol	0.24	0.28	0.26
Clavacin	2.13	2.22	2.05
Streptomycin	3.5	3.1	4.8
Polymyxin	5.6	6.3	7.0
Tyrothricin	7.8	7.2	7.8
Penicillin	8.4	7.2	8.4

[a] Strains of A6 and T11 were virulent strains, and A6-6 was an attenuated strain.

TABLE 2.—*Estimated effective dosages of antibiotics affecting gall tissue of tomato*[a]

Antibiotic	Range tested	ED 50	ED 100
	μg/ml	μg/ml	μg/ml
Effective			
Terramycin	1-115	2	20
Aureomycin	4-115	5	50
Chloramphenicol	1-500	4	125
Moderately effective			
Streptomycin	8-500	60	> 500
Ineffective			
Clavacin[b]	4-60	30	> 60
Polymyxin	12-750	270	> 750
Penicillin	16-2000	420	>2000
Tyrothricin	16-1500	1000	>1500

[a] ED 50 and ED 100 are the concentrations capable of causing 50 per cent and 100 per cent inhibition, respectively.

[b] Clavacin is placed among the ineffective compounds because of its great phytotoxicity.

amphenicol, and polymyxin on marigold were similar to those obtained with tomato.

Subsequent plant experiments of 2 types were devised to determine whether gall inhibition was a result of toxicity to the causal organism or of toxicity to the gall cells. In 1 type of experiment, 2 cultures of the A6 strain of *A. tumefaciens*, 1 resistant to chloramphenicol and the other resistant to polymyxin, were used to infect tomato plants treated with these antibiotics. These cultures were developed by 5 transfers in liquid medium 79 containing successively increased concentrations of the respective antibiotics. The former was able to grow on media containing 40 μg/ml of chloramphenicol and the latter on media containing 300 μg/ml of polymyxin. These levels represented 166 and 53 times, respectively, the concentrations causing 50 per cent inhibition of the parent culture.

Both cultures were altered in their abilities to bring about gall formation. The chloramphenicol- and polymyxin-resistant cultures produced galls with diameters averaging 2/3 and 1/5, respectively, the diameters of those produced by the sensitive parent strain. Neither of these cultures differed from the parent strain in colony characteristics.

In the other type of experiment, bacteria-free gall tissue of marigold was used to test the toxic action of chlormaphenicol, terramycin, and polymyxin on gall cells. The antibiotics were prepared in 4 serial dilutions in sterile distilled water. Five ml was added to each of 6 Erlenmeyer flasks containing 45 ml of the

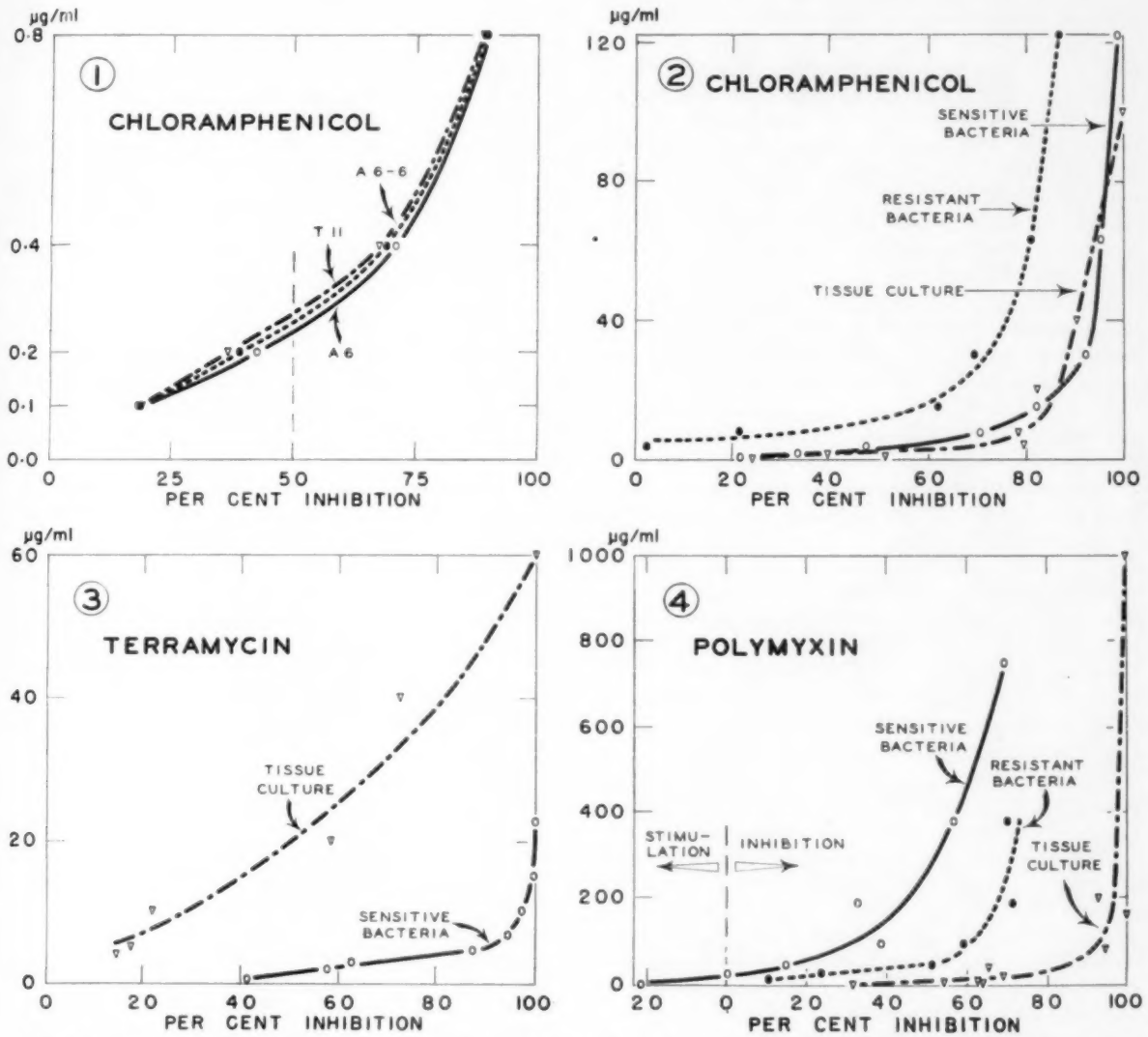

FIG. 1-4.—Fig. 1. Inhibition curves showing action of chloramphenicol on the growth of 3 strains of *A. tumefaciens* in modified medium 79.—Fig. 2. Inhibition by chloramphenicol of crown gall tissue resulting from infection by antibiotic-sensitive and antibiotic-resistant bacteria and of crown gall tissue culture.—Fig. 3. Inhibition by terramycin of crown gall tissue resulting from infection by antibiotic-sensitive bacteria and of crown gall tissue culture.—Fig. 4. Same as Figure 2, inhibition by polymyxin.

TABLE 3.—*Phytotoxic symptoms caused by antibiotics in tomato plants*

Antibiotic	Range tested	Minimum phyto-toxicity	Lethal phyto-toxicity	Bleaching	Wilting	Stunting	Leaf necrosis
	μg/ml	μg/ml	μg/ml				
Terramycin	1-250	30	> 115	slight	severe	severe	moderate
Aureomycin	4-250	50	> 115	slight	moderate	moderate	slight
Chloramphenicol	1-500	60	500	slight	moderate	slight	severe
Streptomycin	8-1000	250	> 500	severe	slight	moderate	moderate
Clavacin	4-125	15	60	none	very severe	slight	slight
Polymyxin	12-750	250	750	none	severe	slight	moderate
Penicillin	16-2000	500	>2000	none	very severe	moderate	severe
Tyrothricin	16-2000	250	>1500	none	slight	moderate	severe

agar medium used by Hildebrandt et al (23). Distilled water was substituted for the antibiotic in the medium of the control flasks. Additions of water and antibiotic were made after the medium was sterilized and before it had hardened. Four pieces of tissue averaging 0.06 g each were transferred aseptically to the surface of the medium in each flask. The tissues were harvested from replicate flasks after 30 days incubation at 27°C and the weights were averaged to a single flask basis. These averaged tissue weights were substituted in equation II and the percentage inhibition of tissue growth by each antibiotic dilution calculated. Growth inhibition curves from triplicate trials were prepared.

Both chloramphenicol and polymyxin caused similar inhibition responses of the bacteria-free gall tissue and of galls on plants inoculated with antibiotic-resistant or antibiotic-sensitive cultures of *A. tumefaciens* (Fig. 2, 4). Terramycin was toxic to gall development in plants and to gall growth in tissue culture, but the inhibition patterns induced were different (Fig. 3).

PHYTOTOXICITY OF THE ANTIBIOTICS.—Injury symptoms were noted for each antibiotic over a wide range of concentrations (Table 3). Because a symptom at any 1 concentration was often masked or nullified by another, various concentrations of each antibiotic were tested so that the maximum expression of each symptom could be obtained. For example, at 125 μg/ml clavacin produced very rapid wilting that caused death of the plants before leaf necrosis became evident. At 15 μg/ml, slight wilting and a slight but definite necrotic pattern were induced.

Typical toxicity patterns on the leaves of tomato plants treated with 6 of the antibiotics are shown in Figure 5. Symptoms caused by aureomycin and tyrothricin resembled those caused by terramycin and penicillin, respectively. These patterns show leaf necrosis except in the case of streptomycin where the pattern shown was induced by bleaching and appeared on the upper part of the plant on the leaves and petioles and to some extent on the main stem. The bleached areas of the petioles and stems showed occasional purplish discolorations, which probably were caused by an unmasking of anthocyanin. Aureomycin and terramycin caused some bleaching of the lower and middle leaves. Chloramphenicol affected the upper leaves slightly.

Higher concentrations of 5 of the antibiotics listed in Table 3 induced moderate to severe plant wilting and often death. Excessive drying and death often followed severe leaf necrosis at higher concentrations of tyrothricin and chloramphenicol.

Stunting occurred with all the antibiotics but particularly with terramycin. Streptomycin and tyrothricin stimulated axillary branching.

ANTIBIOTIC TRANSLOCATION AND STABILITY.—Translocation and stability in tomato plants of the aqueous solutions of terramycin, chloramphenicol, and polymyxin at concentrations of 125, 250, and 500 μg/ml, respectively, were ascertained. Triplicate plants that averaged 12 in. in height and 20 g in weight were treated with 10 ml of these solutions by the root-absorption method. The treated plants were harvested directly after absorption and 30, 60, 96, and 240 hours later. At harvest the roots were washed thoroughly, and the plants were weighed and separated into root, stem, and leaf tissue. Any loss in weight attributed to wilting or drying of the tissues by antibiotic toxicity was restored with distilled water. Finally, each tissue was crushed with mortar and pestle to secure an extract for antibiotic assay. Control extracts were obtained from the nontreated plants.

All plant extracts were assayed by the paper-disc plate method of Loo et al (26). Ten ml of modified medium 79 containing 2 per cent agar was added to each assay plate as the base layer. The surface layer consisted of 5 ml of the same medium containing 1 per cent agar seeded with strain A6 of the test organism. Assays of the extracts were made by adding 0.08 ml of each extract to each of 4 paper discs spaced evenly over the surface of the medium. Duplicate plates were prepared. Root, stem, and leaf extracts were then combined, and the mixture was assayed similarly to obtain an estimate of antibiotic concentration in the total plant tissue. Inhibition zones were measured after 36 hours incubation at 30°C.

Standard curves were obtained by assaying known dilutions of the antibiotics in the same manner. By comparison with these standard curves, the diameters of the inhibition zones obtained with the plant extracts were converted to estimated concentrations of the antibiotic in plant tissue.

This technique was satisfactory for assaying chloramphenicol and terramycin in the plant tissue but was

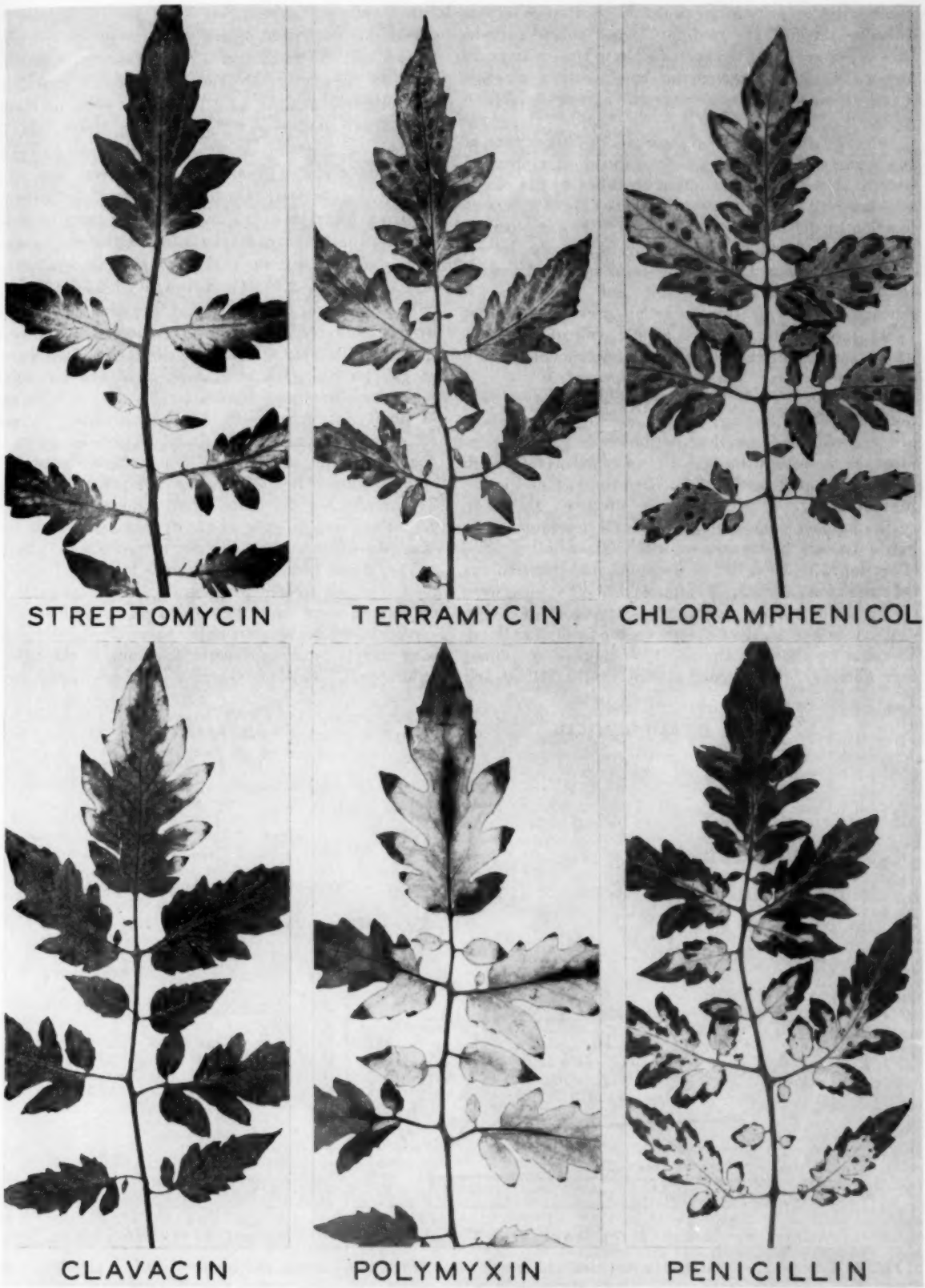

FIG. 5. Symptoms of phytotoxcity by antibiotics on leaves of tomato plants.

inadequate for polymyxin, probably because of its low diffusion rate in agar medium. Some polymyxin was present, however, in the root and stem tissues immediately after plant treatment, since assayed extracts made from these tissues caused narrow inhibition zones.

Directly after absorption, only about 50 per cent of the terramycin and 66 per cent of the chloramphenicol present in the aqueous solution absorbed by the plants were assayed from the tissue extracts (Fig. 6). Translocation of the 2 antibiotics differed. Chloramphenicol moved more rapidly than terramycin into the leaves, and little remained in the roots or stem. Although terramycin also moved rapidly into the leaves, it reached a concentration there no greater than that in the roots. After 10 days, 53 per cent of the terramycin and 20 per cent of the chloramphenicol initially present in the leaf tissue had disappeared.

Discussion.—These results on gall inhibition support the various findings of previous workers. Blanchard (3) obtained complete inhibition of gall development on tomatoes treated by root absorption with 50 μg/ml of aureomycin, the concentration found in the present study to be completely effective. De Ropp (13) obtained some inhibition of gall development on carrot storage tissue treated with 500 μg/ml of penicillin and with 50 μg/ml of streptomycin. Our findings on these 2 antibiotics, in terms of ED 50 values, were of the same order of magnitude as those of de Ropp.

Whether the antibiotic acts on the gall-stimulating bacteria, on the host tissue, or on both is an important question. From comparisons of the ID 50 and ED 50 values (Tables 1, 2), a relationship appears between the sensitivity of the bacteria to various antibiotics and the inhibition of the crown gall development on the plants. Antibiotics introduced into plants and effective against gall formation were inhibitory at concentrations much higher than those that inhibited the bacteria *in vitro*. This seems to be inadequate evidence that the action was primarily against the bacteria. On the other hand, the presence of living bacteria where gall formation was inhibited and the inhibition of bacteria-free gall cells by antibiotics suggest that the action is primarily against the host cells (Fig. 2, 3, 4). Likewise, the inhibition by chloramphenicol and polymyxin of gall tissue induced by antibiotic-resistant bacteria indicates an action on the host cells (Fig. 2, 4). The similarity of the curves of gall inhibition by chloramphenicol and polymyxin in bacteria-free tissue grown *in vitro*, in tissue formed in plants inoculated with antibiotic-sensitive cultures of *A. tumefaciens*, and in tissue formed in plants inoculated with antibiotic-resistant cultures may indicate a common type of antibiotic action in all 3 tests. Terramycin, on the other hand, induced this type of response curve in galls of plants inoculated with antibiotic-sensitive bacteria but not in gall tissue cultures.

The slight bleaching effect noted in the leaves of tomato plants treated with aureomycin, terramycin, or chloramphenicol and the severe effect that was observed in the leaves and upper stem of plants treated with streptomycin apparently were due to the lack of chlorophyll. This toxic action has been noted previ-

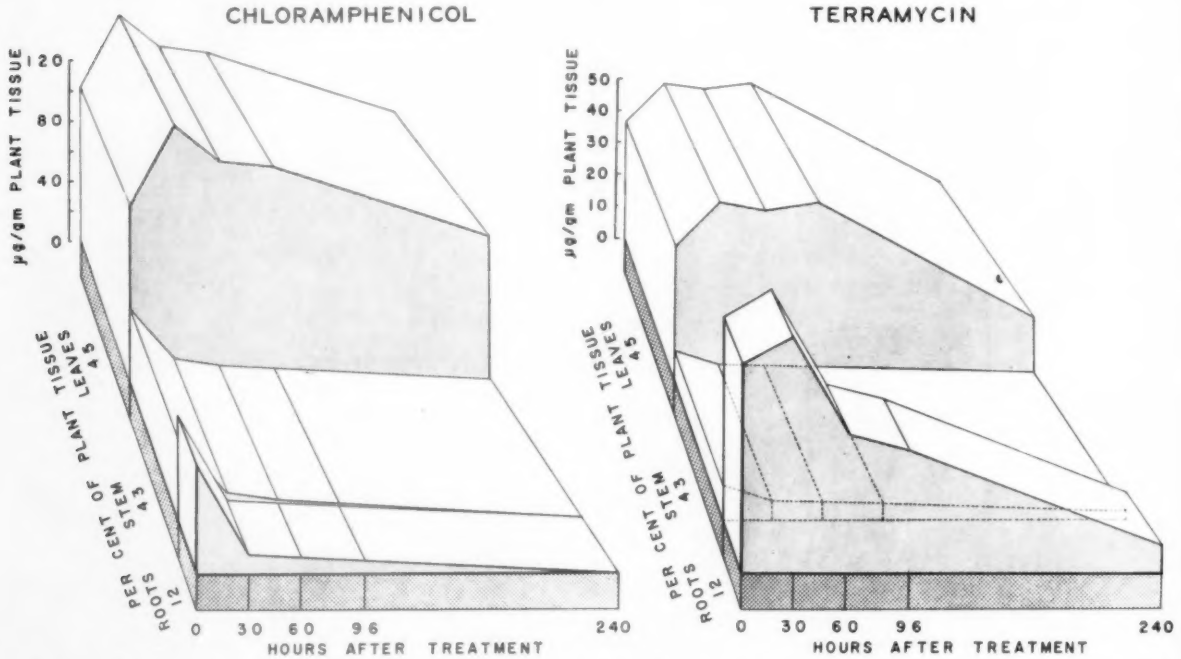

Fig. 6. Concentrations of terramycin and chloramphenicol in the roots, stems, and leaves of tomato plants at stated times after initial root absorption. Plants averaged 20 g wet weight and were treated with 10 ml of aqueous solution containing 125 μg/ml of terramycin or 250 μg/ml of chloramphenicol.

ously in seedlings of various plants treated with streptomycin (5, 19, 32, 34), with aureomycin and terramycin (28), or with chloramphenicol (34).

The rapid absorption, translocation, and comparative stability of terramycin and chloramphenicol in tomato plants suggests the possible value of these antibiotics in the systemic treatments of various plant diseases. The ease with which they are absorbed and translocated, however, undoubtedly varies with the plant concerned. Although, as shown here, both were readily taken up by tomato plants, Pramer (29) found that only chloramphenicol was taken up by the roots of cucumber seedlings.

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THE INHIBITORY EFFECT OF PHENOLIC COMPOUNDS ON THE GROWTH OF STREPTOMYCES SCABIES AS RELATED TO THE MECHANISM OF SCAB RESISTANCE [1]

Lawrence A. Schaal and Gestur Johnson [2]

SUMMARY

The inhibitory effect of 6 phenolic compounds on the growth of *Streytomyces scabies* was tested at pH 6.0, 7.5, and 8.5. Five of these were orthodihydricphenols, and 4 of these (chlorogenic acid, caffeic acid, catechol, and tetrahydroxybenzoin) were found upon autoxidation to be effective in the inhibition of the growth of *S. scabies* in culture medium. The inhibition increased with increases in the pH, which produced an increase in rate of autoxidation. Protocatechuic acid and *p*-hydroxybenzoic acid (a monophenol) were not effective under the conditions of this experiment. The results of this investigation support the theory that the mechanism of scab resistance in potato tubers involves enzymatic oxidation of chlorogenic acid, which produces a quinone toxic to the scab organism.

Chlorogenic acid in the periderm of potato tubers was shown by Johnson and Schaal[3] to play an important role in scab resistance and in the general protective mechanism against injury. The nature of this mechanism is complex and appears to depend on the amount of chlorogenic acid in the periderm and on the presence of tyrosinase in the same tissue. The total amount may not be as important as localization around the lenticels, organs that serve as the normal entrance points for the scab organism. There is a tendency for the acid to accumulate in cells adjacent to injured areas, but the relation of the rate of accumulation of the acid to scab resistance is not clear. Tyrosinase is found also in high concentration in the same tissue as chlorogenic acid and oxidizes it when the tissue is injured. The oxidation products formed, such as quinones, may be toxic to the invading organism. This is in accord with views expressed by Szent-Györgyi and Vietorisz[4] as to the function of tyrosinase when potato tissue is injured. Chlorogenic acid and other orthodihydricphenols that are good tyrosinase substrates increased the rate of formation of suberized tissue when applied to the freshly cut surface of a potato tuber.[3]

It was the purpose of this investigation to determine whether several phenolic compounds upon autoxidation would produce quinones toxic to *Streptomyces scabies* (Thaxt.) Waks. & Henrici in culture medium. Early experiments indicated that chlorogenic acid in the amounts used was not toxic to the races of *S. scabies* tested and that some secondary action was involved. Ingrahm and Corse[5] studied the autoxidation of chlorogenic acid and found a definite relationship between oxygen uptake and hydrogen-ion concentration.

MATERIALS AND METHODS.—Two cultures of *S. scabies* were obtained from scabby potato tubers. Each culture was tested in the greenhouse for pathogenicity, and the degree of parasitism was determined by the depth of the pustule produced on Triumph tubers. Culture S410 produced deep type-4[6] pustules. Culture S463 produced somewhat shallower pustules of type-3. Czapek's agar adjusted to pH 6.2 was used. All plates were seeded by placing 1 ml of spore suspension of *S. scabies* in 15 ml of melted agar and pouring the seeded agar into petri plates. Filter paper discs[7] were placed in the center of the plates on the surface of the agar. Six phenolic compounds were each adjusted to pH 6.0, 7.5, and 8.5 with sodium hydroxide and placed on the test discs in the following amounts: chlorogenic acid, 2, 4, and 6 mg; caffeic acid, catechol, *p*-hydroxybenzoic acid, protocatechuic acid, and tetrahydroxybenzoin[8], 1, 2, and 3 mg. The discs were pressed firmly on the surface of the agar and incubated at 23°C for 4 days when the zones of inhibition were measured. Controls were of 2 types: 1) Czapek's agar adjusted to pH 6.2, 7.5, and 8.5; and 2) Czapek's agar adjusted to pH 6.2, with assay discs impregnated with 0.1 ml of citrate buffer of pH 7.5 or 8 placed on the seeded medium.

RESULTS AND CONCLUSIONS.—The inhibition of growth of *S. scabies* cultures S410 and S463 was more pronounced at pH 8.5 than at 6.0 (Table 1). At pH 6

[1] Accepted for publication July 7, 1955.
Cooperative investigations of Horticultural Crops Research Branch. ARS. U.S. Department of Agriculture and the Colorado Agricultural Experimental Station.
Published with the approval of the Director of the Colorado Agricultural Experiment Station as Scientific Series Paper No. 459.

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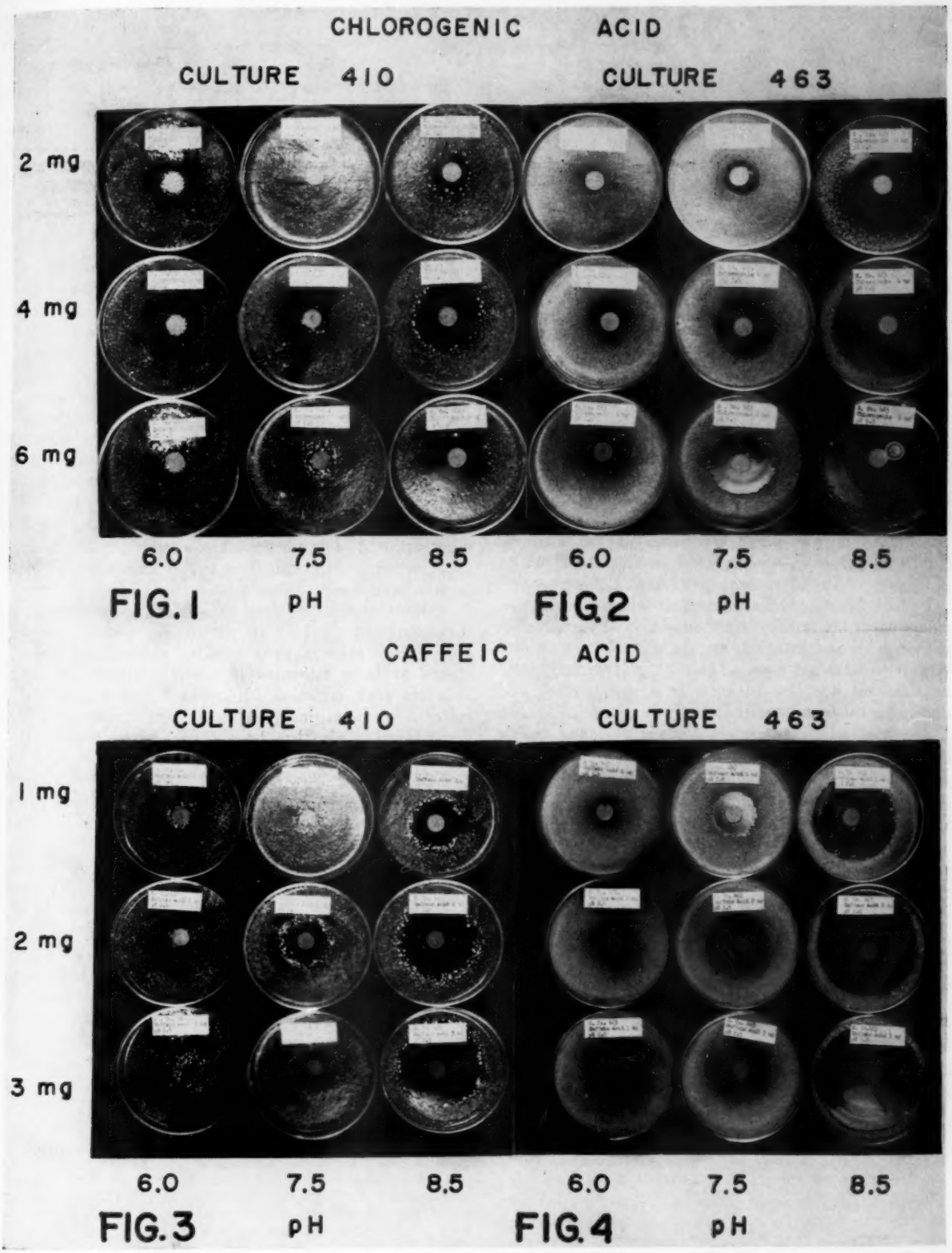
[7] Schleicher and Schuell No. 740. E (12.7 mm).

[8] 3, 4, 3', 4'-Tetrahydroxybenzoin.



FIGS. 1–4. Zones of inhibition produced in 2 cultures of *Streptomyces scabies*, S410 and S463, when 2, 4, and 6 mg of chlorogenic acid and 1, 2, and 3 mg of caffeic acid adjusted to pH 6.0, 7.5, and 8.5 were placed on test discs on the surface of seeded agar plates.

TABLE 1.—*Inhibition of Streptomyces scabies by phenolic compounds adjusted to various pH values*

Phenolic compound	Inhibition of growth[a]					
	Culture S410			Culture S463		
	pH 6.0	pH 7.5	pH 8.5	pH 6.0	pH 7.5	pH 8.5
	mm	*mm*	*mm*	*mm*	*mm*	*mm*
Chlorogenic Acid	0	Trace	10	0	5	18
Catechol	15	15	20	12	12	16
Tetrahydroxybenzoin	12	12	15	5	5	6
Caffeic acid	Trace	11	18	Trace	6	22
p-Hydroxybenzoic acid	0	0	0	0	2	0
Protocatechuic acid	0	0	0	0	0	4

[a] Figures are the widths of the inhibition zones surrounding assay discs.

and 7.5, catechol, tetrahydroxybenzoin, and caffeic acid produced wider zones of inhibition than chlorogenic acid, *p*-hydroxybenzoic acid, or protocatechuic acid. Practically no inhibition by *p*-hydroxybenzoic acid and protocatechuic acid at pH 6.0 and 7.5 was noted, and at pH 8.5 only a small amount of inhibition of culture S463 by protocatechuic acid was noted. No inhibition was noted on either of the controls. Both cultures of *S. scabies* grew readily at pH 6.0 to pH 8.5.

It will be noted that the most effective phenolic compounds, i.e. tetrahydroxybenzoin, chlorogenic acid, caffeic acid, and catechol, are the ones that autoxidize readily to form quinones (Table 2). *p*-Hydroxybenzoic acid, which has low toxicity, is a monophenol and does not oxidize as readily as the orthodihydric phenols.

Zones of inhibition by chlorogenic acid and caffeic acid on cultures S410 and S463 are shown in Figures 1-4. There was more inhibition of culture S463 than of culture S410 at pH 7.5 and at pH 8.5. At pH 6, caffeic acid inhibited growth slightly more than did

TABLE 2.—*Quinone test given by solutions of phenolic compounds after standing 24 hours at 23°C*

Phenolic compound	Quinone test[a]	
	pH 6.0	pH 7.5
Chlorogenic acid 2%	None	Strong
Catechol 1%	Medium	Very strong
Tetrahydroxybenzoin 1%	Weak	Very strong
Caffeic acid 1%	Weak	Very strong
p-Hydroxybenzoic acid 1%	None	None
Protocatechuic acid 1	None	Medium

[a] Acidified potassium iodide-starch solution.

chlorogenic acid. Since the 2 acids were present in equal molar concentration, caffeic acid appears to be more effective than chlorogenic acid. The growth of the fungus near the outer edge of the inhibition zones appeared to be stimulated, perhaps because of the dilution of the inhibitor in this area. Tests for viability of the fungus near the center of the inhibition zones showed that the inoculum was killed by the inhibitor.

Chlorogenic acid in potato tubers has been shown to be important in the resistance of potato tubers to scab and in the general protective mechanism against injury[3]. The results reported herein indicate that upon autoxidation to quinones, chlorogenic acid and other orthodihydric phenolic compounds become toxic to *S. scabies*. Although it is realized that H_2O_2 can be produced upon oxidation of orthodihydric phenols, it is doubtful that any free H_2O_2 was produced under the conditions of this experiment. In potato tubers, chlorogenic acid is enzymatically oxidized by tyrosinase to form quinones in tissues injured by the invading scab organism. It appears that this is the main mechanism involved in scab resistance. The same reaction also has been shown to be associated with suberization of injured potato tissue.

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PEAR BLAST ON VANCOUVER ISLAND [1]

W. E. McKeen [2]

SUMMARY

Pseudomonas syringae v. Hall causes a blossom blight of pears on Vancouver Island, and occasionally multiples so rapidly that a severe epidemic occurs in individual orchards. Although the disease has been present on Vancouver Island for some years, it has been confused with fire blight and physiological disorders. The causal organism was not identified until 1953, and it has not been reported previously on pears in Canada. One unnamed variety of pear proved resistant. *P. syringae* not only blights the pear blossoms, but also causes lesions on leaves, twigs, branches, stems, and fruit. Ooze was not present on the lesions. The pathogen is very sensitive to streptomycin and aureomycin, and very tolerant to penicillin and bacitracin. Streptomycin controls the disease under greenhouse conditions. *P. syringae* grows on agar at 1°C, and may cause canker development during the winter.

During early May in 1953, an epiphytotic of blossom and twig blight of pear occurred on Vancouver Island and affected every orchard to some degree. The severity of the attack ranged from almost no disease in some orchards to a complete destruction of all blossoms, death of buds and twigs in some trees, and even the death of some young trees in other orchards.

For several years, blossom blast has been a very disturbing and limiting factor in the production of pears on the Saanich Peninsula, and according to Hall[3], the disease has been present for more than 20 years. Some agriculturists assumed that it was an atypical fire blight caused by local weather conditions, while others believed it to be a physiological disorder.

In 1947, Jones (3) reported that *Sclerotinia laxa* caused slight to moderate blossom and twig blight on Bartlett, and more especially on Anjou pears, at Sidney, B. C. He also mentioned that he obtained *Botrytis cinerea* from dead twigs, but did not prove the pathogenicity of either organism. A boron deficiency of pears, as described by Woodbridge et al (10) in the Okanagan Valley of British Columbia, causes symptoms similar to those of the disease present in 1953 on Vancouver Island.

Because of the seriousness of pear blast, a study of this disease was undertaken to determine its cause and nature, and possible methods of control.

SYMPTOMS.—Progressive development of the disease permits a description of symptoms in 3 phases: blossom and bud blight, twig blight, and branch and trunk canker.

Blossom and bud blight (Fig. 2, 3), which appears suddenly in early May during the period of full bloom, is the most common phase and frequently is the only phase of the disease that occurs on older trees. In the flower, minute dark spots or bands are first observed on the receptacle. In the very early stages, water-soaking of the tissue is evident at the advancing margins of the lesions, but as the spots rapidly increase in size, they become black and spread until the whole receptacle is involved (Fig. 7). Frequently, an infection spreads down the pedicel for varying distances and may even originate in it.

If a more destructive phase of the disease occurs, the peduncle is blackened, and the whole truss (flowers and leaves) withers. The petals drop, but the affected leaves and flower pedicels and receptacles tend to remain clinging to the spurs for a year. If the flowering spur is only partially killed, new leaves may emerge, and thus leaves in 2 distinct stages of development may appear on diseased trees.

Buds frequently are attacked (Fig. 2, 6) and killed and as a result there are regions devoid of leaves or fruit on branches of diseased trees.

In younger trees, a brown discoloration of the cambium and phloem (Fig. 8) often extends from diseased peduncles and buds several inches along the stem beyond a point that shows no external evidence of infection. In a few weeks, the parenchymatous and periderm layers outside the necrotic tissue become dry and depressed, and then the limits of the lesions (Fig. 9, 11) are clearly marked by this depression and also by a crack that frequently forms at the margins of the lesion. Cankers may enlarge considerably throughout the winter season, and as a result young trees may be killed. In a local nursery, lesions appeared on branches of 4 per cent of the trees.

Microscopic examinations showed that a *Cytospora* sp. and another secondary fungus (Fig. 10) appear on some of the dead twigs several months after the tissue becomes necrotic.

Other observations of possible significance have been made. Gum or exudate never has been observed on any of the cankers or diseased organs of the pear

[1] Accepted for publication July 18, 1955.
Contribution No. 1464 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.
Acknowledgment is extended to Dr. W. R. Ashford of Merck & Co., Ltd., Montreal, Quebec, for supplying streptomycin and assaying its presence in pear fruit. The assistance of Misses Audrey Linnes and Carole Valens and Mr. M. C. J. Van Adrichem is deeply appreciated.
[2] Plant Pathologist.
[3] Letter from E. R. Hall, Assistant Superintendent, Experimental Station, Saanichton, British Columbia. January 25, 1955.

tree. Marginal lesions and black necrotic spots occur on leaves (Fig. 5), especially of the Bosc variety, and lesions have been found on the fruit in July. Varietal resistance to blossom infection has been

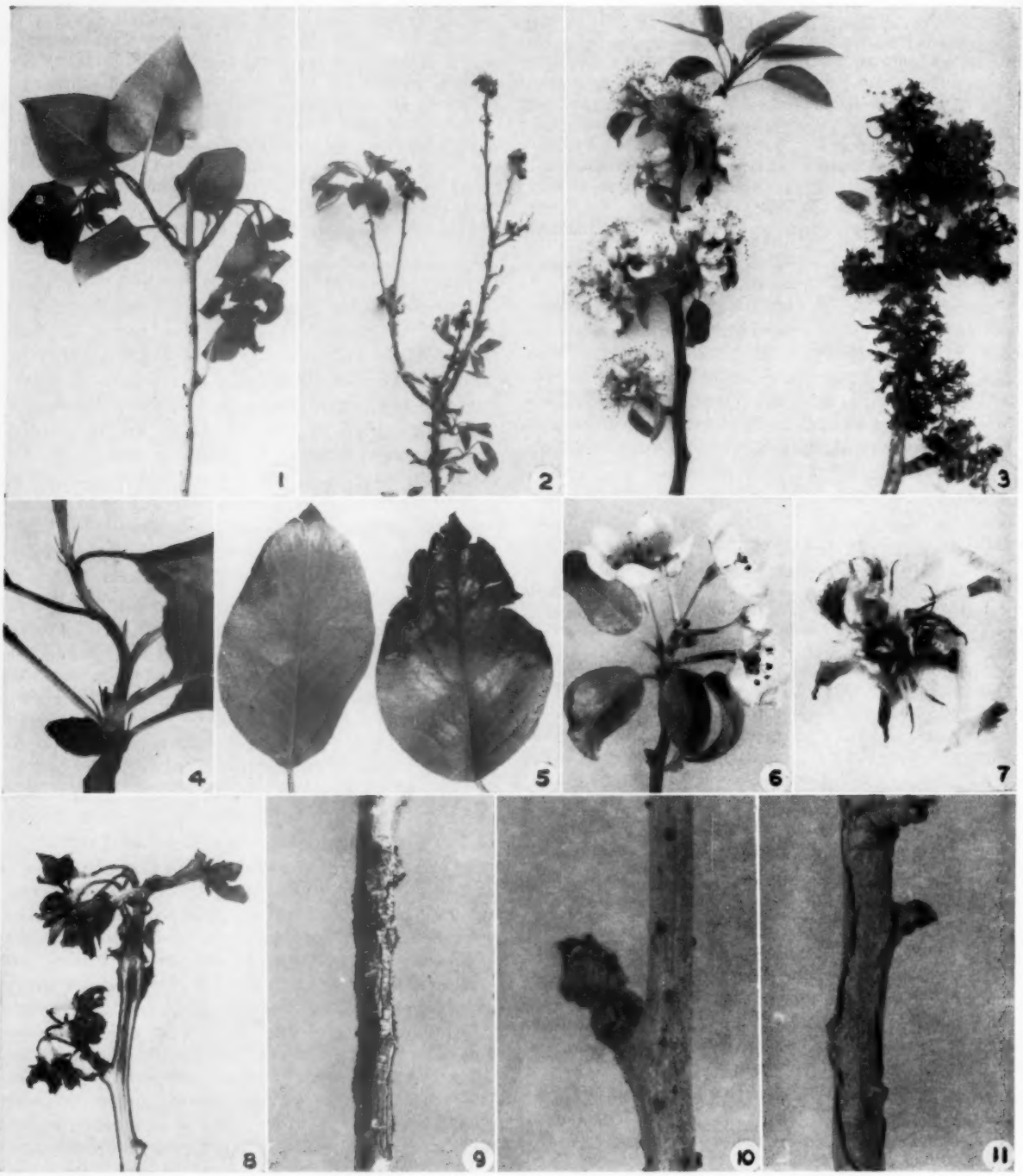

FIG. 1-11.—Fig. 1. An infected lilac branch.—Fig. 2. Top of a tree with blasted buds and flowers.—Fig. 3. Two pear branches: left, with healthy blossoms, right, with blasted blossoms.—Fig. 4. A young succulent pear stem that was inoculated with *P. syringae*. Observe that it has become black and that necrosis has spread into the leaf petiole.—Fig. 5. Leaves with black lesions.—Fig. 6. A flower truss with an infected bud. The whole truss will die as a result of a single infection.—Fig. 7. A flower with a black receptacle, caused by infection.—Fig. 8. A tangential section along the cambium of a diseased pear twig. Note that the cambial region is necrotic, but the cortex is white.—Fig. 9. A canker on the stem of a young tree.—Fig. 10. A diseased twig that shows the fruiting bodies of a secondary organism.—Fig. 11. A pear lesion that has spread down the stem from the upper infected bud.

observed in a top-worked Olivier de Serre tree; the variety of the scion is unknown. Only an extremely small amount of infection occurred in cherry, plum, and apple trees in plantings adjoining heavily infected pear orchards.

ISOLATION OF CAUSAL ORGANISM.—As the pear blast occurred on trees to which complete fertilizers and minor elements such as zinc, boron, and manganese had been applied, it seemed improbable that the disorder was of a nutritional nature. The appearance and development of the disease suggested that it was of bacterial origin, and microscopical examination of blighted tissues revealed an abundance of bacteria. Dilution plates made from diseased material in the spring of both 1953 and 1954 yielded in almost every instance bacterial colonies of similar appearance. Fifteen cultures were selected for inoculation experiments and cultural studies. Two of these cultures were isolated in February, 1 from a bud and the other from a branch canker. Cultures also were obtained from lilac, plum, cherry, and apple trees.

STUDY OF PATHOGEN.—When blossoms, succulent twigs, young leaves, and green fruit were punctured with a needle and then sprayed with a suspension of the pear-blight organism, typical blight symptoms appeared in several experiments. In the fruit, the bacteria spread through the vascular system and into the pedicel, and the fruit dropped within 10 days after inoculation. A bacterium that proved to be identical with that used for inoculation was easily isolated from the inoculated tissue. It was evident that the pathogen was not *Erwinia amylovora* (Burr.) Winsl. et al (Table 1).

On yeast-extract agar, all isolates produced colonies that were smooth, glossy, circular, undulate, low convex, and opaque. The colonies were more convex and somewhat larger on potato-dextrose agar. Nutrient agar supported only slight growth that was filiform, transparent, and glossy. On this medium, a faint green, fluorescent pigment was produced, which became darker and brown with age. This green fluorescence was very distinctive in Clara's medium (2).

TABLE 1.—*Comparison of pear-blight organism with Erwinia amylovora*

Properties	Pear-blight organism	*Erwinia amylovora*
Dimensions	0.3-1.0 × 1.0-1.5μ	0.75-1.2 × 1.2-2.0μ
Flagellae	2 to 5 polar	Peritrichous
Thermal death point	51° C	47° C
Reaction on Clara's medium[a]	Green pigment	No pigment
Reaction on gelatin	Stratiform liquefaction	Uniform liquefaction
Reaction on litmus milk	Alkaline peptonized, litmus reduced	Acid to alkaline, litmus reduced
Condition of broth	Turbid	Turbid with pellicle
Type of growth on agar slant	Butyrous, opaque	Moist, opaque
Occurrence of ooze on host	Absent	Present
Pathogenicity on lima and kidney bean	Positive	Negative

[a] 0.3 g $MgSO_4$, 2.0 g K_2HPO_4, and 3.0 g asparagine per liter. Adjusted to pH 6.5.

The isolates did not produce nitrites from nitrates, grow in a 5 per cent salt solution, produce indol, or hydrolyze starch. The isolates weakly fermented 5 per cent sucrose in beef extract, and a small amount of acid but no gas was produced. Evidence that acid was being produced was obtained within 3 days when bromthymol blue was used as an indicator. The minimum, optimum, and maximum temperatures for growth were 1°, 26°–30°, and 40°C, respectively. The isolates from pear, lilac (Fig. 1), plum, apple, and cherry did not differ significantly from each other in morphology, physiology, and behavior in culture. From the description of *Pseudomonas syringae* v. Hall given in *Bergey's Manual of Determinative Bacteriology* (1), the isolates appeared to be identical with that organism. All isolates produced typical symptoms on pear seedlings and lilac bushes. The author, unlike Rosen (8) in tests with *P. syringae*, was unable to obtain infection on lemon fruit with the isolates used in the present study.

Those isolates were identical with a culture of *P. syringae* isolated from pear in California[4], but a plum isolate of *P. syringae* from California[5] produced a blue color, whereas the author's isolates produced a yellowish green in Wilson's (9) peptone-free medium containing lactose and bromthymol blue. When lactose was replaced by sucrose, the plum culture turned the medium green, whereas the others turned it yellow.

The above results indicate that the pear disease is caused by *P. syringae*.

With the use of sensitivity discs, it has been determined that the bacterium is very sensitive to streptomycin and aureomycin (7-chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide), is very tolerant to penicillin and bacitracin, and responds in an intermediate fashion to terramycin (4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide), chloromycetin (D(−)-threo-2,2-dichloro-*N*-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl]acetamide), and polymyxin. *P. syringae* does not grow in a broth solution containing 1 ppm of streptomycin nor on agar containing 50 ppm of streptomycin.

CONTROL.—In order to avoid initial infection, it is essential that healthy stock be planted, and advisable to remove wild host plants from the adjoining area.

Because of the multiplicity of infections that occur

[4] Culture obtained from Professor P. A. Ark, University of California, Berkeley, California.

[5] Culture obtained from Profesor W. H. English, University of California, Berkeley, California.

in most diseased trees, it is hardly practicable to prune out the cankers. Antibiotic dusts and sprays may prove to be effective, but the lack of blight in the orchard that was treated during the spring of 1954 made it impossible to form conclusions regarding their efficacy. Pear trees have not shown any toxic symptoms when 7 sprays containing 500 ppm of crude streptomycin were applied at weekly intervals. Assays showed that streptomycin was not present in the fruit in August. The tests were carried out by a microbiological test that indicated the presence of streptomycin down to the ratio of 0.1 μg of streptomycin in about 300 g of fruit.

In the greenhouse, lesions were produced on the tender stem tissues of vigorously growing seedlings when they were inoculated with a needle 2 days after a streptomycin spray of 400 ppm had been applied. When young succulent stems (Fig. 4) and leaves were wounded before a streptomycin spray at 400 ppm was applied, however, no infection occurred when a broth solution of *P. syringae* was sprayed on the trees after the antibiotic spray had dried.

Discussion.—The most destructive bacterial disease of pear trees in North America is fire blight, but the author has not observed it on Vancouver Island during the last 3 years. Instead, there has occurred a blossom-blast disease, which is always present to some extent in all orchards and in some years is epidemic in some orchards. Except on young trees, infection usually is limited to the tissue of the current year's growth. The blasting occurs with astonishing suddenness.

The similarity between the syndrome of the blasting of pears on Vancouver Island and the boron disease of pear described by Johnson et al (6) in southwestern Washington is very striking. The correlation in Washington between summer rainfall and incidence of disease, however, does not hold for Vancouver Island. Also, large limbs are never killed on old trees.

The fact that pear blast has not been reported elsewhere in Canada is rather interesting, since the pathogen has been found on lilac in Ontario, Quebec (4), and Nova Scotia (5). The exact cause of this freedom from pear blast is not known. Temperature would not appear to be a limiting factor, as *P. syringae* is capable of attacking succulent pear tissue over a wide range of temperatures.

The duration of the blossoming period on Vancouver Island is several days longer than in many other regions in Canada, and thus the host is susceptible for a much longer period of time. Nevertheless, this disease has been reported by Parker and Burkholder (7) on pear in New York State where conditions are quite similar to those in parts of eastern Canada.

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A DISEASE OF ORCHARDGRASS CAUSED BY PLEOSPORA PHAEOCOMES [1]

J. H. Graham

SUMMARY

A previously undescribed foliar disease of orchardgrass was studied and the causal agent identified as *Pleospora phaeocomes*. The asexual stage was a Helminthosporium related to the *H. gramineum* group but distinct from previously described species. In greenhouse inoculations of the common forage grasses, only orchardgrass became infected. A less widely grown grass, *Holcus lanatus*, was mildly attacked. Optimum temperature for mycelial growth was approximately 20°C. The present paper is the first report of the occurrence of *P. phaeocomes* in the United States and the first demonstration of its pathogenicity.

A previously undescribed disease of orchardgrass (*Dactylis glomerata* L.) caused by a species of *Pleospora* with a Helminthosporium as the asexual stage has been under observation since 1953. The fungus has been isolated from leaves and sheaths of orchardgrass plants in Central Pennsylvania as well as in the southern tier counties (2). Although the disease is not prevalent, the fact that plants are killed back to the crown in greenhouse inoculations indicates that it is a potential economic threat. Results of studies of the disease and its causal agent are reported in the present paper.

SYMPTOMATOLOGY.—In nature, the disease is evidenced by marginal, light brown, irregular lesions up to 15 mm long and 5 mm wide and by blighted leaves. A few scattered conidiophores develop on the necrotic tissue, and black perithecia may be seen underneath the epidermis. When diseased leaves are placed in a moist chamber, several conidia develop on each conidiophore. On overwintered leaves, the large perithecia break through the epidermis and can be seen without magnification.

Young lesions on artifically inoculated leaves are yellow to orange, round or oblong, 1–4 mm in diameter, and often have watery borders. Later the centers become necrotic as the lesions enlarge, coalesce, and finally kill part or all of the leaf.

THE PATHOGEN.—The fungus sporulates sparingly on host tissue. Dark brown conidiophores arise singly, or very rarely in groups of 2 or 3, between epidermal cells or through stomates and are 103–232μ (to first scar) $\times$ 6–11μ, with 5–8 septa.

On undisturbed tissue, 4–6 conidia may develop on a conidiophore before being detached. Spores are light brown to greenish-brown, straight, widest usually at the second cell, and slightly tapered toward the tip (Fig. 1, A). The basal cell may be lighter and have thinner walls than the other cells; often it is separated by a slight constriction. Conidia are 42–83μ $\times$ 10–17μ, usually 50–70μ $\times$ 11–15μ, with 4–6 septa. The peripheral wall is thin and includes a large, conspicuous hilum within its contour. Although germination may occur from any of the cells, germ tubes normally arise from the end cells only. The asexual fruiting structures of the fungus are somewhat similar to the species of the *H. gramineum* complex (3) but can be distinguished from previously reported species of *Helminthosporium* (*H. sativum* Pam., King, & Bakke, *H. triseptatum* Drechs., and *H. victoriae* Meehan & Murphy (5) on orchardgrass and also from other fully described species of the genus.

Mature perithecia have been found in the spring on overwintered leaves of orchardgrass and on artificially inoculated plants that had been left in the field over winter. They are 600–850μ $\times$ 400–620μ, black, erumpent, and slightly flattened, with a short broad beak. Dark brown septate setae, up to 300μ long, arise from the upper portion of the perithecium. On artificially inoculated leaves, conidia may be borne on some of the bristles from immature perithecia. The scleroid perithecium consists of a thick inner wall of parenchymatic cells enclosed by a thin black rind. Pseudoparaphyses can be seen among the asci. The asci, measuring 200–260μ $\times$ 40–52μ, are slightly clavate with a

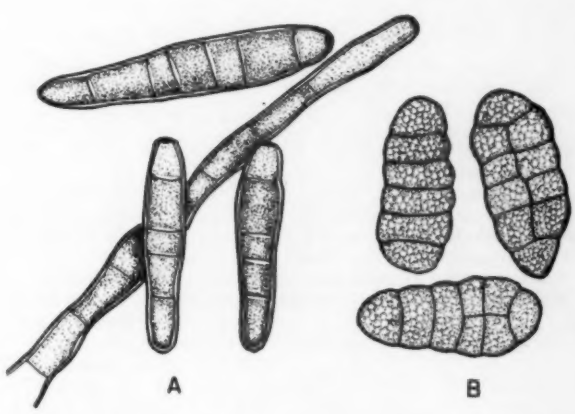

FIG. 1. Spores of *Pleospora phaeocomes* ($\times$ 470). A) Conidiophore and conidia. B) Ascospores.

[1] Accepted for publication July 21, 1955.
Contribution No. 146 of the U. S. Regional Pasture Research Laboratory, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, State College, Pennsylvania, in cooperation with the 12 Northeastern States. The author wishes to thank Dr. L. E. Wehmeyer for assistance in identifying the fungus.

thick wall, particularly at the apex.

Ascospores are biseriate at first but become uniseriate as the asci elongate in water. The spores are oblong-ellipsoid, yellow to orange, and 41–67μ × 20–26μ. In collections made at 4 locations in Pennsylvania, mature spores have been consistently 5-septate (Fig. 1,B). In the development of the ascospores, a single midwall is first formed, later the 2 cells divide, and finally 2 more walls are formed between the latter and the midwall. Normally, a vertical wall extends across 2 cells but may be lacking or extend across 1, 3, or 4 cells. Vertical septa have not been observed in the end cells. The spores are constricted at all septa, being more so at the junction of cells with vertical septa.

The fungus produces abundant mycelium but few conidia on potato-dextrose agar. Mycelium is first white, changing with age to light then dark gray and becoming black underneath. Colonies are raised, fluffy, and even margined. Conidia for inoculations were produced by partially drying mycelial mats that had grown in potato-dextrose broth, and then placing them in a moist chamber for 3 days at room temperature (20°–23°C).

Mature perithecia were produced on Sach's agar when cultures were alternated several times from 5°C to room temperature for a period of 4 months. The fruiting bodies were 400–600μ, submerged, round to slightly flattened, and with or without a few short setae. The ascospores were smaller than those found in nature and were 3- to 7-septate. Perithecia containing ascospores similar to those found in nature were produced on sterilized orchardgrass stems held at alternating temperatures.

Maximum mycelial growth in potato-dextrose broth occurred at approximately 20°C. At temperatures above 25°C, growth was greatly retarded.

In greenhouse inoculations, single conidial and ascospore cultures were equally pathogenic on orchardgrass but failed to attack several of the common forage grasses. Seedlings of *Holcus lanatus* L. were mildly attacked. A few irregular chlorotic and necrotic lesions developed on the blades, and some of the leaf tips were killed.

IDENTIFICATION OF THE PATHOGEN.—The fungus under study belongs in the pseudosphaeriaceous group of pyrenomycetes. The presence of setae on the perithecia places it in the genus *Pyrenophora* as described in much of the literature. Wehmeyer (6) and others believe, however, that the setose condition is not constant enough to justify generic rank. In a later paper, Wehmeyer (8) presents evidence that the name Pyrenophora should be discarded or moved to the Fungi Imperfecti because of the sterile nature of the type material.

The fungus on orchardgrass is similar to *Pleospora trichostoma* (Fr.) Ces. & de Not., as described by Wehmeyer (7), except that the ascospores are 5-septate instead of 3-septate. This condition would place it in the complex group identified as *Pleospora phaeocomes* (Reb.) Wint., although ascospores from European collections are larger than those described in the present paper. *P. phaeocomes* has not been reported previously from the United States. Fuckel (1) appears to have been the first to describe ascospores of this species; he lists them as 72μ × 20μ with 3, and later many, septa. He also found conidia of Helminthosporium on the host, *Holcus lanatus*, which he describes only as being similar to *P. relicina* (Fr.) Wint. The latter species is synonymous with *P. trichostoma*. Müller (4) described *Pyrenophora phaeocomes* on *Calamagrostis varia* (Schrader) Host with still larger ascospores, 90–105μ × 33–39μ, than those listed by Fuckel. In addition, a collection of this species with 6-septate ascospores on *Anthoxanthum* sp. was sent to Wehmeyer from Sweden (8).

The present paper constitutes the first record of *P. phaeocomes* as a pathogenic organism.

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PHYTOPATHOLOGICAL NOTES

Cotyledonary Inoculation, a Method for Screening Spinach for Blight Resistance. R. E. WEBB.[1] Spinach blight caused by cucumber virus 1 is a limiting factor in spinach production in certain areas. Incorporation of resistance to this disease in superior varieties and hybrids involves screening of large numbers of spinach seedlings. Early elimination of susceptible seedlings would result in selection and hybridization of only those plants resistant to the disease.

Seeds of the varieties Virginia Savoy (resistant) and Bloomsdale (susceptible) of *Spinacia oleracea* L. were planted in pots at 24°C. When the cotyledons were approaching mature size, 30 plants of each variety were dusted lightly with carborundum, and the cotyledons were inoculated with the virus that causes spinach blight. Inoculum obtained from stock cultures of the virus maintained in plants of *Nicotinia rustica* L. was applied with sterilized cheesecloth. A second series of the 30 plants of each variety was inoculated as the cauline or first true leaves were reaching maximum size. All plants of the variety Bloomsdale developed symptoms irrespective of the type of leaves inoculated. Two Virginia Savoy plants inoculated through the cotyledons and 5 inoculated through the cauline leaves became infected. Pound and Cheo[2] found that this variety is more resistant at 16°–20° than at 24°–28°C. Consequently, 45 Bloomsdale plants and 34 Virginia Savoy plants were inoculated through the cotyledons at 15°–20° in 2 separate trials. In 29 days, 42 of the 45 plants of Bloomsdale were diseased, whereas all inoculated plants of Virginia Savoy remained healthy. Attempts to recover the virus from the Virginia Savoy plants by tests on cowpea were negative.

Bloomsdale, when inoculated through the cotyledons, developed symptoms concomitantly with the appearance of the cauline leaves. The results indicated that susceptible seedlings in a segregating population could be eliminated 2–4 weeks earlier by the cotyledonary inoculation method than by the usual method of inoculating the cauline leaves.

Two hundred forty-nine plants of an F_2 population of 3 parental combinations that were segregating for resistance to blight were inoculated in the cotyledonary stage and held at 16°–20°C for 32 days. All plants that developed blight were removed, and the remaining plants were reinoculated with the virus. Later the plants were transplanted to 6-in. pots for observation. Eight additional plants became diseased. A similar procedure was used in evaluating an F_2 population of 149 plants of 7 parental combinations. On reinoculation, 2 of the plants that appeared resistant developed symptoms.

Preliminary results indicate that at 16°–20°C, infection of plants with the virus causing spinach blight does not affect seedling reaction to *Peronospora effusa* (Grev. ex. Desm.) Ces., the cause of blue mold. This method makes it possible to screen segregating populations simultaneously for resistance to both blight and blue mold.—Plant Industry Station, Beltsville, Maryland.

Relative Susceptibility of Guatemalan and Mexican Avocado Rootstocks to Verticillium Wilt. GEORGE A. ZENTMYER, F. F. HALMA, AND STEPHEN WILHELM.[1] Verticillium wilt of the avocado tree (*Persea americana* Mill.) has been recognized as such since 1949, when isolation of the causal fungus (*Verticillium alboatrum* Reinke & Berth.) and proof of its pathogenicity were reported.[2] The disease apparently had been present in California for a number of years prior to 1949, as the symptoms described by Horne in 1934[3] for "asphyxiation, apoplexy, or collapse" are very similar to those of Verticillium wilt. This disease is not at present a serious problem in avocado production in California. Data presented in this paper, however, indicate that the disease could become a serious factor under coastal soil-temperature conditions with a wider use of the more susceptible rootstocks.

Until recently, little information has been available on the relative susceptibility of various rootstocks to this disease. The California avocado industry is based on budded or grafted trees, with Mexican seedlings as the primary rootstock source, although some Guatemalan seedlings and Guatemalan-Mexican hybrid seedlings have been used. In the original description of the disease,[2] it was noted that after artificial inoculation Guatemalan seedlings showed symptoms earlier and were more severely affected than Mexican seedlings.

Experimental avocado rootstock plots established near Santa Barbara, California, in 1948 by Halma and White[4] have made it possible to obtain information on

[1] Plant Pathologist, Horticultural Crops Research Branch, Agricultural Research Service, United States Department of Agriculture.

[2] Pound, G. S., and P. Cheo. 1952. Studies on resistance to cucumber virus 1 in spinach. Phytopathology 42: 301-306.

[1] Respectively, Plant Pathologist, Department of Plant Pathology, and Professor of Horticulture (Emeritus), Department of Horticulture, University of California Citrus Experiment Station, Riverside; and Associate Plant Pathologist, Department of Plant Pathology, University of California, Berkeley.

[2] Zentmyer, G. A. 1949. Verticillium wilt of avocado. Phytopathology 39: 677-682.

[3] Horne, W. T. 1934. Avocado diseases in California. Calif. Agr. Exp. Sta. Bul. 585, 72 p.

[4] Halma, F. F., and F. A. White. 1948. Avocado rootstock trials in Santa Barbara County. Calif. Avocado Soc. Yearbook 1948: 103-107.

the relative susceptibility of different rootstocks to Verticillium wilt. These plantings are in a coastal region, with a cool mild climate, and include 14 varieties of Guatemalan and 5 varieties of Mexican rootstocks planted in random fashion. A Guatemalan scion (MacArthur variety) was used on all of these trees.

Verticillium wilt has now appeared on trees in 3 of the 7 rootstock plots in Santa Barbara County. Symptoms of the disease are similar to those previously described.[2] In the experimental plots, the foliage wilting on diseased trees has ranged from 25 to nearly 100 per cent. Recovery is common after the initial wilting and dieback. Recovery of some trees is apparently complete, no disease recurrence having taken place 4 years after the initial symptoms appeared. Other affected trees "recovered," but wilted again in subsequent years.

In plot 1, 18.5 per cent of 92 trees on Guatemalan rootstocks were infected by 1955, whereas 2.8 per cent of 72 trees on Mexican rootstocks were infected. In plot 2, the figures were 10 per cent of 70 and 2 per cent of 49, respectively. In plot 3, 2.4 per cent of 85 trees on Guatemalan rootstocks were infected, but none of the 107 trees on Mexican rootstocks were diseased.

In the first 2 plots, either the land had been planted to tomatoes prior to its use for avocado culture, or tomatoes had been used as interplants between the rows of young avocado trees. Culture of an extremely susceptible crop such as tomato would be expected to increase the inoculum potential of *V. albo-atrum* in the soil and thus be responsible for the high percentage of disease in these avocado plantings. The practice of growing tomatoes in association with avocados has been discouraged since the discovery of the cause of the disease.

Inoculations with *V. albo-atrum* made by Wilhelm in glasshouses of the University of California at Berkeley confirm the greater susceptibility of Guatemalan rootstocks. Seeds were germinated in sand, and the root systems of the seedlings were dipped into a spore suspension of the fungus at the time the seedlings were transplanted into pots of soil. Seedlings were inoculated in March and April, 1953, and the first disease symptoms appeared approximately 1 month after inoculation. Of the 30 Guatemalan seedlings inoculated, 22 became infected in the period of the experiment (Table 1). Only 9 of the 30 inoculated Mexican seedlings became infected.

TABLE 1.—*Number of infections following inoculation of seedlings on Guatemalan and Mexican rootstocks with Verticillium albo-atrum*

Rootstock and variety	Number of seedlings		
	Inoculated	With symptoms	Infected[a]
Guatemalan			
Dickinson	10	8	8
Nabal	12	9	9
Anaheim	8	5	5
Mexican			
Mexicola	9	4	4
Ganter	10	2	3
Topa-Topa	11	0	2

[a] Based on cultures of vascular system.

Results of these observations and inoculations indicate that although both Guatemalan and Mexican avocado rootstocks are susceptible to infection by *V. albo-atrum*, the Guatemalan varieties are appreciably more susceptible than the Mexican.—Departments of Plant Pathology and Horticulture, University of California Citrus Experiment Station, Riverside, and Department of Plant Pathology, University of California, Berkeley.

Incidence of Tobacco Mosaic Virus on Tomato Seed. C. A. JOHN[1] AND CARL SOVA[2]. Tobacco mosaic is one of the most prevalent diseases of tomato regions. The present study was initiated to ascertain the source of infection in commercial fields in Ohio. Attempts to recover the virus from the hands of workers who use tobacco, from composted soil in which plants were grown, and from the weeds growing in the vicinity were uniformly unsuccessful. Tests for tobacco mosaic virus were made on the local-lesion host, *Nicotiana glutinosa* L.

In 1949, Alexander[3] reported that virtually all brands of cigarettes and a large percentage of other kinds of tobacco were carrying the virus. In the present studies, water extracts from 7 popular brands of cigarettes and 5 kinds of cigars failed to produce lesions on the test host in June, 1953. Later, 2 extracts from single cigarettes from each of 7 brands produced 1 and 6 lesions. The following week, 10 cigarettes from each of 2 packs of these brands were assayed. None of the 20 extracts produced lesions.

Jones and Burnett[4] found that tomato seed from plants affected with mosaic did not produce any virus-infected seedlings, whereas studies in England[5] and in Canada[6] show that tobacco mosaic virus may be carried on the seed. Doolittle[7] states, "In commercial practice, however, where the seed has been dried for some time before planting, there is little if any seed transmission of virus."

Plants of *N. glutinosa* were grown in 6-in. pots until 4–8 nearly mature leaves were formed. One g of

[1] Head, Crop Research Department, H. J. Heinz Co., Pittsburgh, Pennsylvania. P. O. Address, H. J. Heinz Co., Bowling Green, Ohio.

[2] Formerly Research Horticulturist, Crop Research Department, H. J. Heinz Co.; now graduate student, Ohio State University, Columbus, Ohio.

[3] Alexander, L. J. 1949. Control of the mosaic of glasshouse tomatoes. Ohio Vegetable and Potato Growers' Assoc. 34th Ann. Proc. p. 128-138.

[4] Jones, L. K., and G. Burnett. 1935. Virous diseases of greenhouse-grown tomatoes. Wash. Agr. Exp. Sta. Bul. 308, 36 p.

[5] Bewley, W. F., and W. Corbett. 1930. The control of cucumber and tomato mosaic diseases in glasshouses by the use of clean seed. Ann. Appl. Biol. 17: 260-265.

[6] Berkeley, G. H., and H. O. Madden. 1933. Transmission of streak and mosaic diseases of tomatoes through seed. Sci Agr. 13: 455-458.

[7] Doolittle, S. P. 1948. Tomato diseases. U. S. Dept. Agr. Farmers' Bul. 1934, 83 p.

TABLE 1.—*Recovery of tobacco mosaic virus from 1952 tomato seed stored 4 months in common storage then in small envelopes in the laboratory*

Variety	Months storage in laboratory	Number of samples	
		Tested	With virus
Rutgers Regular	0	4	2
	½	6	4
	1	6	5
	1½	6	4
	2	3	3
	3	1	1
	4	3	3
	5	3	3
	6	3	1
	7	3	0
Rutgers Nursery	0	2	2
	½	2	2
	1	2	1
	1½	2	2
	2	1	1
	3	1	1
	4	1	1
	5	1	1
	6	1	0
	7	1	0

tomato seed to be assayed was placed in a sterilized mortar with 10 ml of distilled water and thoroughly crushed with a pestle. A few drops of this solution were then applied with absorbent cotton on the upper surface of a leaf previously dusted with 600-mesh carborundum. Five minutes after the inoculation was completed, the leaf was rinsed with distilled water. The host plants were maintained at 80°F or above. Lesions appeared in 36–48 hours and usually were counted after 72 hours, but in a few cases where lesions were few, counting was postponed until 96–120 hours.

In assays made in 1953, virus was found on lots of 3 different varieties of regular tomato seed saved during 1950, 1951, and 1952 and held in bags in common storage. Although there was a variation in the amount of virus present, there was no indication of a great loss of infectivity during storage.

Small seed samples removed from large bags of seed and stored in paper envelopes under laboratory conditions were assayed bimonthly during February and March and then monthly until October, 1953. Under these conditions, the virus apparently disappeared by the end of the test period (Table 1). It is postulated that the more rapid inactivation of the virus in this test than in that where seed was kept in common storage was the result of greater exposure to air in the envelopes than in the bags.

The work of Brock[8], which indicated that trisodium phosphate is effective in inactivation of the virus, was confirmed. In this case, the seed of the Rutgers Regular was treated with a 10 per cent solution of trisodium phosphate for 15 minutes. Repeated germination tests in soil indicated this treatment will not cause any dimunition in viability for treatment periods up to and including 4 hours' duration, whereas a 20 per cent solution was not injurious at periods up to and including 2 hours. Results indicated a reduction in emergence ranging from 5 to 30 per cent for prolonged treatments at both the 10 and 20 per cent concentrations.—Crop Research Department, H. J. Heinz Co. Pittsburgh, Pennsylvania.

A Winter Rye Useful in Identifying Soil-borne Wheat Mosaic Virus[1]. W. H. SILL, JR.[2] The necessity for maintaining cool temperatures and short daylengths to obtain satisfactory symptoms[3] has been a distinct disadvantage in the greenhouse study of soil-borne wheat mosaic virus, *Marmor tritici* Holmes emend. McK.[4] In 1953, winter rye plants (*Secale cereale* L.) that showed severe green mosaic symptoms late in the season even after the onset of hot weather were found growing in virus-infested soil of a field in Johnson County, Kansas. The variety, although not known, was thought to be Balbo. Seed was collected from these plants. In 2 years, seed of this selection has been planted in virus-infested soils from both Kansas and Illinois.[5] Each year the plants have been kept at average winter greenhouse temperatures, 70°F or more, with no attempt to control daylength. Excellent green mosaic symptoms have developed in plants grown in both soils. The long incubation periods characteristic of this disease on wheat[3] also have been typical of the disease on this rye (69 days or more from planting date). Seed is being increased and will be furnished as available to those interested.—Kansas Agricultural Experiment Station, Manhattan.

Longevity of Ustilago nuda in Barley Seed. R. H. PORTER. In the course of breeding work with barley carried on cooperatively by the Departments of Agronomy and Botany and aimed at the development of varieties resistant to the loose smut pathogen, *Ustilago nuda* (Jens.) Rostr., many varieties have been planted and tested. Artificial inoculation of individual heads was made each year but natural infection was not unusual.

In 1953, a planting was made with seed from non-

[8] Brock, R. D. 1952. The use of trisodium phosphate as an inactivating agent for plant viruses. Jour. Austral. Agr. Sci. 18: 41-43.

[1] Contribution No. 598, Serial No. 459, Department of Botany and Plant Pathology, Kansas Agricultural Experiment Station, Manhattan.

[2] Assistant Professor of Botany and Plant Pathology, Department of Botany and Plant Pathology, Kansas State College, Manhattan.

[3] McKinney, H. H. 1937. Mosaic diseases of wheat and related cereals. U. S. Dept. Agr. Circ. 442.

[4] Koehler, B., W. M. Bever, and O. T. Bonnett. 1952. Soil-borne wheat mosaic. Ill. Agr. Exp. Sta. Bul. 556.

[5] Infested Illinois soil furnished by Dr. W. M. Bever, Department of Agronomy, Illinois Agricultural Experiment Station, Urbana.

inoculated heads harvested in 1942 from the variety Coast I. The plants that grew from this particular lot of seed produced several heads that appeared to be affected by loose smut. Chlamydospores from 1 head, when tested for germination, produced a promycelium with no sporidia, a condition typical for spores of *U. nuda*. It seems evident, therefore, that the loose smut fungus remained viable in barley seed embryos for a period of 11 years.

In this connection it is of interest to refer to past records on the longevity of spores of loose smut fungi. Stakman[1] in 1913 reported that spores of *Ustilago tritici* kept in a dry place from July 8, 1909, to December 10, 1909, remained viable, and spores of *U. nuda* remained viable in dry storage from July, 1909 to April, 1910. Spores from heads of barley and wheat, each infected with loose smut fungi, when buried in pots in soil in 1909 and kept outside during the winter germinated the following spring.

Dickson and Johann[2] have kept spores of *U. nuda* in gelatine capsules stored at 4°C for 6 months with no loss in viability. At temperatures above 8°C they found that viability was retained for not more than 30 days.

The loose smut pathogen remains in the scutellum of barley seeds in the form of a fungous hypha that apparently is a much more stable condition than is the form of chlamydospores. It is well known that the low relative humidity of the atmosphere and moderate summer temperatures in the vicinity of Fort Collins provide exceptionally favorable conditions for the longevity of seeds and the same may well apply to certain seed-borne fungi carried internally.—Colorado Agricultural Experiment Station, Fort Collins, Colo.

[1] Stakman, E. C. 1913. Spore germination of cereal smuts. Minn. Agr. Exp. Sta. Tech. Bul. 133.

[2] Dickson, J. G., and Helen Johann. Unpublished data furnished the writer in correspondence, July 19, 1955.

LISTING OF BOOKS

PHYTOPATHOLOGY will, from time to time as space becomes available, list titles of books on pathological subjects. A Brief description of the contents of the books will be included.

FREAR, D. E. H. 1955. **Pesticide handbook**. Ed. 7. College Science Publishers, State College, Pa. 208 p. Price, paper $1.25, cloth $3.00.

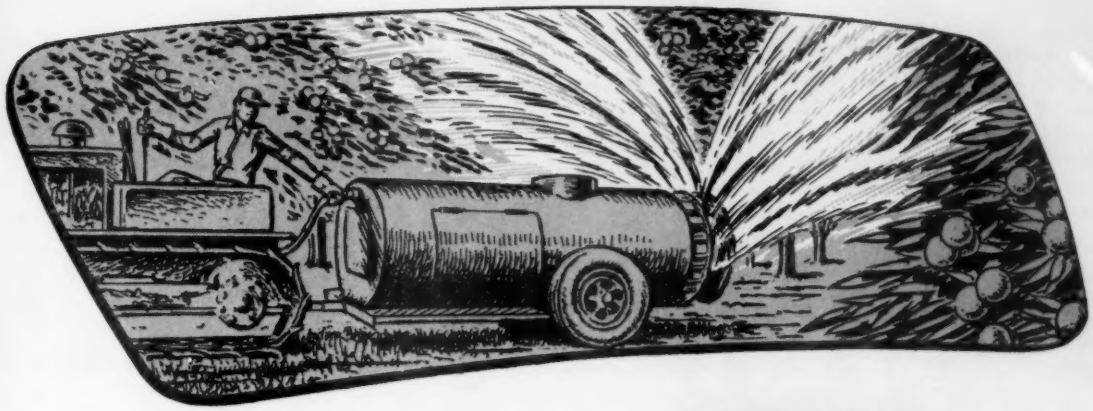
A total of 6,204 commercial pesticides are listed alphabetically by name. The percentage active ingredients and the manufacturer are given in each case. Separate alphabetical listings give use and ingredients, and manufacturers.

NIGHTINGALE, GORDON T. 1955. **Horticultural science.** Horticultural Publications, New Brunswick, New Jersey. 111 p.

The material presented has been used at Rutgers University in a laboratory course in horticultural science for undergraduate students who have had plant physiology. Each chapter begins with a concise discussion of the subject under consideration, and this is followed by laboratory exercises, questions, and references. In the presentation, the physiology of the plant as a whole is considered, with carbohydrates and proteins taken as the chief points of reference.

BARNETT, H. L. 1955. **Illustrated genera of imperfect fungi.** Burgess Publishing Co., Minneapolis, Minnesota. 218 p. Price $4.00.

Following a key of 15 pages, 302 genera of the Fungi Imperfecti are illustrated by pen drawings and described briefly. An alphabetical index concludes the text. The book should be a handy reference to anyone interested in this large group of pathogens and saprophytes.



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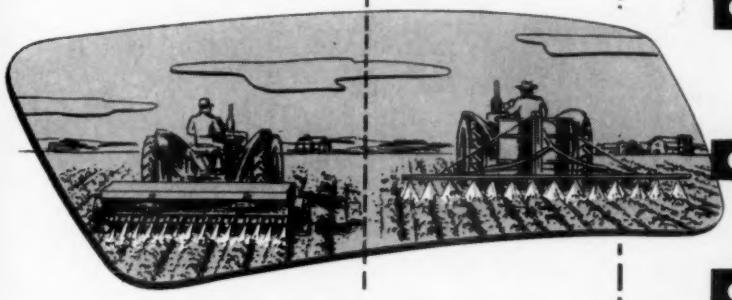

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NEWS

Phytopathologist Wilfred D. Mills of Cornell University was presented a U. S. D. A. Superior Service Award for 32 years of outstanding extension work in New York. Dr. Mills was cited "for outstanding success in interpreting detailed research to practical fruit growers, resulting in extreme accuracy in pest control and in increased quality in fruit and overall grower satisfaction and income."

An honorary life membership in the Potato Association of America was conferred on Karl H. Fernow at the annual meeting in East Lansing. Dr. Fernow was honored for over 33 years of devotion to the problems of potato production, especially the production and use of high grade certified seed.

The Canadian Agricultural Chemicals Association held its third annual meeting and conference at Ste-Adele-en-haut, Quebec, on October 13 and 14.

Visiting plant Pathologist Leonard Broadbent of the Rothamsted Experiment Station, England, has been conducting research at the Connecticut Agricultural Experiment Station. Professor Broadbent is an authority on transmission of virus diseases by insects.

A sister organization, the Indian Phytopathological Society, reports that its membership for 1954 was 188.

Virologist Robert E. Wilkinson of Cornell University is conducting research on citrus problems at the Rehovet Experiment Station. Dr. Wilkinson will be in Israel for 12 months and plans to return next June.

After 30 years' service George H. Coons will retire November 30 from the U. S. D. A. Dr. Coons is well known for his contributions to sugar beet pathology and breeding. He will continue as Assistant Head of the Sugar Crops Section and devote some time to writing.

Deaths: Harry B. Humphrey formerly Principal Pathologist, Cereal Division, U. S. D. A. died October 13 at the age of 82. Dr. Humphrey was President of the Society in 1945 and served as Editor-in-Chief of Phytopathology for a number of years.

Editor: K. W. KREITLOW, Plant Industry Station, Beltsville, Md.

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